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TESTING EXPERIMENTAL COMPOUNDS AGAINST LEISHMANIASIS IN LABORATORY ANIMAL MODEL SYSTEMS

ANNUAL/FINAL REPORT

Janet S. Keithly, Ph.D.

April 1988

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3039

Cornell University Medical College
New York, New York 10021

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Cornell University Medical College; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): New York, New York 10021	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-83-C-3039
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62770A	3M1-62770A870	AM	009

11. TITLE (Include Security Classification)
(U) Testing Experimental Compounds Against Leishmaniasis in Laboratory Animal Model Systems

12. PERSONAL AUTHOR(S)
Janet S. Keithly

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final	FROM 12/15/82 TO 12/14/86	1988 April	70

16. SUPPLEMENTARY NOTATION
Annual repts 1984-1986
Final rept. 15 Dec 82 - 14 Dec 86

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Leishmania donovani; L. b. braziliensis; BALB/c mouse
L. m. mexicana; Crithidia fasciculata; J774.1N- cells
L. braziliensis guyanensis; Trypanosoma b. brucei; →

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

A. Pentostam Dose Response

Pentostam Dose Response for L. donovani, L. m. mexicana, and L. braziliensis guyanensis were determined in BALB/c mice using amastigotes and promastigotes. In each test system, primary culture promastigotes were more resistant to treatment than other stages. The Effective Dose 50 for these cells in each test system was 290, 21, and 6 mg/kg/day. Cutaneous infections caused by L. mexicana were 3 to 7X more difficult to treat than were L. donovani or L. b. guyanensis. This is due to the genetics of the parasite, host immune response, and drug used.

B. Experimental WRAIR Compounds

Seven of 10 WRAIR 1984 compounds suppressed L. donovani 79 to 94%. Although it was highly probable that these drugs would be competitive with Pentostam, none of them were available for further testing. The remaining three compounds were toxic, and were not competitive with antimonials.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mary Frances Bostian	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

19. Key Words (continued from Block 19)

Amastigote	Combination Chemotherapy	Trypanothione Reductase
Promastigote	Ketoconazole	Gene Sequence
Allopurinol riboside	Suramin	Synthetic Probes
Difluoromethylornithine	Sterols	RNA Virus (LR1)
Berenil	Polyamines	Sandfly
Melarsen oxide	Trypanothione	Species-specific
Pentostam	Ornithine Decarboxylase	Cytocidal/Cytostatic

20. Abstract (continued from Block 20)

C. Combination Chemotherapy with difluoromethylornithine (DFMO)

DFMO inhibits in vitro growth and in vivo replication of L. donovani and L. braziliensis guyanensis, but not L. mexicana. The effect of DFMO alone upon L. b. guyanensis is striking. This is the first time an oral, non-toxic, specific alternative to antimonials or Amphotericin B. has been identified which is active against experimental mucocutaneous leishmaniasis.

Depending upon the species, DFMO can be synergistic, additive, or have no effect when combined with other drugs. DFMO and the antitumor antibiotic Bleomycin synergistically suppress experimental infections of L. donovani. When used in combination with Suramin, Allopurinol riboside, Berenil, or Pentostam, DFMO had an additive effect against visceral leishmaniasis in the mouse model.

In vitro data confirmed these in vivo effects. DFMO significantly reduces putrescine and trypanothione levels in leishmania. Since leishmania depend upon polyamine and trypanothione metabolism for survival, these pathways should be actively pursued as potential targets for chemotherapy.

The pentavalent antimonial Pentostam does not inhibit the biosynthesis of trypanothione in vitro.

D. Azoles

The de novo biosynthesis of species-specific plasma membrane sterols by amastigotes of L. braziliensis and L. mexicana indicates that this is a possible area for the development of chemotheapy. However, species show differences in sterol biosynthesis. This could account for the differences in sensitivity of patients to treatment with the antifungal drugs ketoconazole, allylamines, and Amphotericin B.

E. Molecular Aspects of Chemotherapy

The gene for ornithine decarboxylase (ODC) in Leishmania has been isolated using a cloned probe from Trypanosoma b. brucei. This is the gene for the rate-controlling enzyme in polyamine biosynthesis. Expression of this gene is currently being studied with and without drug pressure in order to understand the species-specific differences in susceptibility to DFMO. The gene is also being sequenced.

The gene for trypanothine reductase (TR) is currently being isolated from Leishmania using synthetic probes based upon the amino acid sequence from a non-pathogenic flagellate, Crithidia fasciculata. This is the gene for the controlling enzyme in trypanothione biosynthesis. It is being examined for expression with and without drug pressure.

F. Virus in Leishmania braziliensis subspecies

An RNA virus (LR1) has been isolated and characterized from L. b. guyanensis CUMC 1 1-A. Its effects on the parasite are unknown, but its origin is probably from the sandfly vector. Since LR1 is a potential transformation vector for Leishmania, it may serve as a way to target nonsense codons into the parasite, and to disrupt its life cycle. Perhaps antiviral agents should be explored as potential treatment for mucocutaneous leishmaniasis in combination with other drugs.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for Care and Use of Laboratory Animals" prepared by the Committees on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH 86-83, Revised 1985)].

SUMMARY

I. Years 1982 - 1984

A. Pentostam Dose Response

Pentostam Dose Responses for L. donovani, L. m. mexicana, and L. braziliensis guyanensis were determined in BALB/c mice using amastigotes (A), primary culture promastigotes (PCP), and subcultured promastigotes (SP). In each test system, PCP were more resistant to treatment than were A or SP. This is biologically significant, since PCP are equivalent to the infective stage from sandflies. The Effective Dose 50 (ED 50) for PCP in each secondary test system were 290, 21, and 6 mg/kg/day for L. m. mexicana, L. b. guyanensis, and L. donovani Khartoum, respectively. A detailed chart can be found in Annual Report 2, DAMD17-83-C-3039, page 4.

These data showed that Pentostam caused improvement and parasitological cures in WRAIR primary (hamster), secondary (BALB/c), and tertiary (owl monkey) test systems at doses and regimes equivalent to those used to treat humans. The validity of each of these models in drug screening to predict the outcome in human infections was established.

Cutaneous infections caused by L. m. mexicana are 3 to 7X more difficult to treat than are L. donovani or L. b. guyanensis infections. This is due partially to the genetics of the parasite, immune response of the host, and probably drug delivery to tissue sites.

B. Experimental WRAIR Compounds

Seven of 10 WRAIR 1984 compounds suppressed L. donvani 79 to 94% at the MTD, MTD/2, and MTD/4. Although it was highly probable these drugs would be competitive with Pentostam, none of them were available for further testing. The remaining three compounds were variously toxic, and therefore not competitive with Pentostam.

For 6 of 7 drugs active against L. donovani, Pentostam Indices of 4.3 to 84.0 occurred against L. b. guyanensis when measured as percent suppression from controls. None of these drugs suppressed lesions from their original size. All drugs were delivered orally as per change in protocol by 1983 COTR. The lack of efficacy against cutaneous lesions of 1984 drugs was most likely a problem of drug delivery to site. None of these 7 drugs were available for additional testing.

C. Combination Chemotherapy of Pentostam with DFMO

A synergistic effect of the polyamine inhibitor DFMO and the antibiotic Bleomycin was documented against L. donovani

infections in BALB/c mice. This was the first time in 30 years a new drug competitive with Pentostam showed efficacy against visceral leishmaniasis. Further testing showed suppession of L. b. guyanensis infections in this animal model when 2% DFMO was used in combination with Pentostam, and 5% DFMO was used alone. Later experiments during the final two years of this contract demonstrate that Pentostam in combination with DFMO is additive in its effect against visceral and mucocutaneous infections, but not effective against cutaneous L. m. mexicana infections. The biochemical and molecular basis of these species-specific differences is remarkable, and is apparently true for many drug groups tested.

D. Evaluation of L. braziliensis braziliensis models

During the first two years of this contract, we showed that L. b. braziliensis M2904, the World Health Organization type specimen, had special requirements for growth and transformation in vitro. Although we had established experimental infections in BALB/c mice with this, and a more recent isolate from Peru (CUMC 3), the revisions of this contract for the remaining two years precluded drug screening in this model.

II. Years 1984 - 1986

The original scope of this contract was modified due to the paucity of experimental WRAIR compounds for testing in the animal model systems. Following a meeting in July, 1984, the revised scope of DAMD17-83-C-3039 for years 1984-1987 would be:

A. Testing efficacy of DFMO in combination with known and promising antileishmanial drugs including allopurinol, its riboside, Berenil, Pentamidine, Pentostam, Suramin, and other drugs first in the visceral model. If promising combinations were discovered, then to test these in the cutaneous and mucocutaneous models.

B. To determine the probable biochemical mode of action of DFMO, Pentostam, and inhibitors of sterol biosynthesis against L. donovani, L. m. mexicana, and L. b. guyanensis in vitro.

C. To begin to study the molecular basis of the mode of action of these new drugs in order to understand how resistance develops, and to design better drugs.

D. To determine the basis of virulence in L. braziliensis subspecies, and to study its implications for chemotherapy.

A. DFMO inhibits in vitro growth and in vivo replication of L. donovani and L. b. guyanensis, but not L. mexicana. The effect of DFMO alone upon L. braziliensis guyanensis is striking. This is the first time an oral, non-toxic, specific alternative to antimonials or Amphotericin B has been identified which is active

against experimental mucocutaneous leishmaniasis.

Depending upon the species, DFMO can be synergistic, additive, or have no effect when combined with other drugs. DFMO and the antitumor antibiotic Bleomycin synergistically suppress experimental infections of L. donovani. When used in combination with Suramin, allopurinol riboside, Berenil, or Pentostam, DFMO has an additive effect against experimental visceral leishmaniasis.

B. Polyamine and Trypanothione Metabolism. Our in vitro data confirm these in vivo effects, and coincided with the discovery of trypanothione, a compound involved in leishmania glutathione metabolism. Trypanothione reductase (TR) is the rate-controlling enzyme for maintaining significant amounts of reduced glutathione (GSH) in leishmania. TR levels in both amastigotes and promastigotes of Leishmania is high. Promastigotes of species of Leishmania contain high levels of GSH, glutathionyl-spermidine (GSH-SPD), and $T(SH)_2$. Each of these is essential for glutathione metabolism and cell survival. DFMO significantly reduces GSH-SPD, $T(SH)_2$, putrescine and spermidine levels in leishmania.

These DFMO-induced changes in metabolite levels are similar to those reported for trypanosomes. The effect of DFMO alone would appear to be cytostatic rather than cytocidal. Although the precise mechanism by which DFMO induces cytostasis is unknown, it must be due to a depletion of polyamines and $T(SH)_2$, because its effect can be completely reversed by putrescine. These data suggest that it may be necessary to combine DFMO with a cytocidal drug like Bleomycin or Suramin to effect a complete cure.

Since Leishmania and other trypanosomatids depend upon polyamine and trypanothione metabolism for survival, we think these should be actively pursued as potential targets for chemotherapy.

B. Sterol Biosynthesis. The promastigotes of L. braziliensis guyanensis and L. mexicana contain cholesterol, derived from the medium, and synthesize 5,7 C_{28}-sterols. Ergosta-5,7, 22-trien-3 beta-ol is the major product of L. b. guyanensis, but in L. m. mexicana a different ergosterol predominates. There is no evidence of 5-C_{28} sterols in promastigotes. Therefore, there are species-specific differences in plasma membrane sterols of cutaneous and mucocutaneous species of Leishmania. This has significant implications for the design of specific drugs against plasma membrane sterols of leishmania.

Amastigotes of these two species of Leishmania contain cholesterol and desmosterol. These are products of the host macrophage. The amounts of 5,7-C_{28} sterols found in promastigotes are diminished in amastigotes, but a new class of C_{29} stigmasterols appears and accounts for about 20% of the total sterols in L. m. mexicana amastigotes. In addition to 5-C_{28} sterols, both L. m. mexicana and L. b. guyanensis amastigotes contain new classes of ergosterols not seen in promastigotes.

There are again, species-specific differences in the types of ergosterols synthesized. In L. braziliensis guyanensis, the efficiency with which two new ergosterols is synthesized from host macrophage desmosterol is remarkable. This may account for differences in drug sensitivity of these two species to inhibitors of sterol biosynthesis (ketoconazole, allylamines).

The de novo biosynthesis of species-specific, fungus-like plasma membrane sterols by amastigotes of L. braziliensis and L. mexicana indicates that this is a promising area for the development of new chemotherapy. However, these biochemical data also indicate that the efficacy of new compounds will vary according to the way in which each species synthesizes sterols. This could account for the differences in sensitivity of patients with cutaneous and mucocutaneous leishmaniasis to the antifungal drugs ketoconazole, itraconazole, and even Amphotericin B.

C. We have identified the rate-limiting enzyme for polyamine biosynthesis, ornithine decarboxylase (ODC), in Southern blots of L. braziliensis guyanensis by using a cloned probe of ODC from Trypanosoma b. brucei. We are currently isolating this gene from cDNA libraries of L. mexicana and L. donovani. Once the gene is isolated, we plan to compare the sequences for ODC from these species, in order to see whether there are structural or functional differences which can account for the species-specific differences in susceptibility to treatment with DFMO.

The gene for trypanothione reductase (TR) from Leishmania species is currently being isolated. A number of synthetic oligonucleotides have been constructed for TR, based upon the partial amino acid sequence for the redox active site of this enzyme from Crithidia fasciculata, a non-pathogenic kinetoplastid, sequences of other flavin oxidoreductases, and codon usage in Leishmania. Primer extension studies have enabled us to determine the sequence for C. fasciculata 123 nucleotides 5' to the redox active site. This has enabled the construction of additional synthetic probes. Primer extension studies using RNA from three genera of kinetoplastids, i.e. Crithidia, Leishmania, and Trypanosoma, indicate that the former two genera make extension products using the synthetic probes, whereas the latter does not. This indicates that there may be very real differenes in this enzyme between genera, and this may be important between and among species. If so, this could have profound implications for designing drugs or vaccines against species of Leishmania.

D. Two subspecies of Leishmania braziliensis contain small nucleic acids that have the properties expected for self-replicating, acquired genetic elements. One of these, LD1/CD1, is a DNA that appears to exist either integrated into chromosomal DNA or in a circular form. This resembles a plasmid or virus. The other is an RNA virus (LR1) which was discovered in Leishmania braziliensis guyanensis CUMC1, a human pathogen recently isolated from a patient who travelled to Surinam. LR1 is about 6000 nucleotides, single-stranded, and restricted to the cytoplasm. No

homologous LR1 sequences are detected in L. b. guyanensis genomic DNA. Sequence analysis of a cDNA clone of this virus indicates that it encodes a protein homologous to the L protein of vesicular stomatitis virus.

The effects of LR1 on the parasite are not yet known. To date, there is no correlation between the presence of LR1 and any disease characteristic, host range, or biochemically distinct metabolite produced by L. b. guyanensis. Although the origin of the virus is unknown, sandflies are also known vectors for RNA viruses.

Since LR1 is a potential transformation vector for Leishmania, it may serve as a way to target nonsense codons into leishmania and to disrupt the life cycle. Or, since the virus is present within L. braziliensis subspecies perhaps antiviral agents should be tested in combination with other drugs, like DFMO, in patients with mucocutaneous disease.

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I. Objectives

To serve as a secondary screen for promising WRAIR experimental compounds against cutaneous, mucocutaneous and visceral leishmaniasis in BALB/c mice. Specifically, to test 10 compounds a year against Leishmania mexicana mexicana, L. braziliensis panamensis (or guyanensis), and L. donovani, and to test 5 compounds a year against L. braziliensis braziliensis.

The original scope of this proposal was twice modified chiefly due to a paucity of promising experimental drugs identified in the hamster primary test system against cutnaeous and mucocutaneous leishmaniasis. A summary of the discussions to modify the scope of this proposal is found on page 10 of the 1984 September Annual Report 2. Briefly, it was recommended that:

A. Ten experimental WRAIR compounds be tested in the Leishmania donovani BALB/c model;
B. A Pentostam Dose Response be completed for each model;
C. Combination chemotherapy using alpha difluoromethyl-ornithine (DFMO) + Pentostam against all three species of Leishmania model systems be started as a new direction; and
D. Basic research on the L. b. braziliensis BALB/c test system continue for one year.

In addition, it was decided that these recommendations and new directions for the contract be discussed further with the incoming COTR Major James Lovelace. At a preliminary meeting in April, 1984, the PI presented several new directions for consideration including: 1) combination of difluoromethylornithine (DFMO) with known and promising antileishmanial drugs, 2) mode of action of Pentostam in vivo and in vitro, 3) use of carnitine analogs to inhibit leishmania, 4) use of iron chelators to interfer with heme-iron acquisition by leishmania, and 5) significance of iron siderophores for virulence of L. brziliensis subspecies.

Following a meeting with WRAIR COTR Lovelace in July, 1984, it was decided that the final revised scope of DAMD17-83-C-3039 for the remaining years would investigate:

A. Efficacy of DFMO in combination with known and promising antileishmanial drugs, including Allopurinol, Berenil, Pentamidine, Pentostam, and Suramin in the visceral and cutaneous animal models, and if successful, in the mucocutaneous model;
B. Determine the probable mode of action of these drugs in vitro and in vivo if identified;
C. Begin to study the molecular basis of the mode of action of these drugs, in order to understand how resistance develops and to design better drugs; and
D. To study virulence factors in L. braziliensis which might impact on chemotherapy of mucocutaneous disease.

II. Background

Human leishmaniases are still severely debilitating worldwide, affecting about 1 billion people (127). In addition to the World Health Organization, the public health importance of leishmaniasis has been recognized by the United States NIH NIAID through its increased funding of several Collaborative Program Projects for Training in Tropical Disease. In the Americas, leishmaniasis occurs as cutaneous, mucocutaneous, or visceral disease from the Southern United States to Northern Argentina (5,57,72,79,107,129). There are 10-15 million cutaneous and 400,000 visceral cases are reported annually (31,79,102).

A recent update estimates several million of the new cases yearly, are due to fulminant disease in HIV positive patients (10). For example, five patients seen at CUMC for consultation, who had developed acute visceral leishmaniasis (VL) lived outside endemic areas (10,16,28). They either had HIV infection or a clinical syndrome compatible with AIDS. Three diagnosed as HIV positive by Western blots died of acute fulminant VL while living in Spain (16). The fourth died of overwhelming VL in spite of several courses of Glucantime therapy. A fifth had both HIV and VL, but had not been in an endemic area for several years. These cases demonstrate the potential for HIV to convert asymptomatic leishmaniasis into fatal disease, which is unresponsive to convential (antimony) therapy. As HIV infections spread, additional patients can be expected to present with this disease.

In addition to these new cases of VL among civilians, cutaneous leishmaniasis (CL) has been a problem among U.S. Army personnel (118) and in British troops (33,76) in Central and South America, as well as Israeli (88) and now Soviet troops in the Middle East (B.H. Kean, pers. comm.). In light of events in all of these areas, and the continued interest of the United States in the security of civilians and military personnel, it becomes imperative that improved therapy against the leishmaniases be found.

Chemotherapy of leishmaniasis is still based upon rather toxic compounds, arsenicals and antimony derivatives. Since our last report, these three drug groups have not changed: pentavalent antimonials (Pentostam), diamidines (Pentamidine), and macrolide antibiotics (Amphotericin B). Unfortunately, however, species of Leishmania causing VL, CL, and mucocutaneous leishmaniasis (MCL) resistant to pentavalent antimony and to the macrolide antibiotics have been reported (59,91,97). In some cases, this was induced gradually, a situation likely to occur during less than optimal treatment regimes (59), but naturally occurring cases of resistance whose cause is unknown have been also been reported (13,19,23,31,100,120). Several mechanism of resistance have been documented for species of Leishmania and

their relatives, the Trypanosoma. Two of these are: gene amplification which results in overproduction of the target enzyme (15,46,66), or changes in the permeability of the parasite membrane to the drug (12,29,63).

Fortunately, in addition to their medical significance, Leishmania species exhibit a number of properties, different from most mammalian cells [separation of the glycolytic pathway into glycosomes, presence of a modified DNA-rich mitochondrion, the kinetoplast, unusual thiols and lipids, and sterol metabolism resembling that of fungi (35,52,109,124)], which may offer new avenues of therapy.

Attempts to understand the mechanism of action of these compounds, resistance to them, and to identify unique metabolic targets unique to leishmania have resulted in a number of new, and promising leads for the treatment of leishmaniasis. Much of this work has been done by our laboratory as a new direction for this contract during the last two years. The emphasis has been upon understanding the biochemical basis of Leishmania chemotherapy in order to design new, more specific drugs, and to employ combination chemotherapy against metabolic pathways specific for their survival.

III. New Targets for Chemotherapy

A. Polyamine and Trypanothione Biosynthesis

Much of our biochemical understqnding of Leishmania pathways and their significance comes from studies on the African trypanosome, Trypanosoma b. brucei, and the monogenous insect kinetoplastid Crithidia fasciculata. Our recent studies, detailed under the results sections, clearly show their importance in Leishmania species.

We had mentioned previously (Final Report, DAMD17-80-C-0061) the synergistic effect of difluoromethylornithine (DFMO)/Bleomycin against L. donovani infections in BALB/c mice, and had recommended the testing of other known and promising antileishmanial compounds with it. We report here the efficacy of DFMO in combination with a wide range of known or experimental antileishmanial compounds, including: allopurinol, allopurinol riboside, 9-deazoinosine, Berenil, Pentamidine, Pentostam, and Suramin. Depending upon the species of Leishmania, DFMO in combination with these drugs had an additive, synergistic, or no effect. In vivo results correlated remarkably with in vitro results. During the course of this work, the effect of DFMO upon two biochemical pathways, polyamine and trypanothione biosynthesis, was elucidated. **These data indicate that in leishmania, and other kinetoplastids, the two pathways are interdependent, and represent specific targets for chemotherapy in these protozoa (Addendum 1;** 38,68).

1. Biochemical and Metabolic Aspects

a. Background.

Thiols and their reduction are important for the survival of Leishmania species. Glutathione is a major thiol-containing compound in trypanosomatids (3.5 nmol/mg protein), and it is the major reductant used by them to rid themselves of H_2O_2 and peroxide-related free radicals, since they lack catalase and have low levels of superoxide dismutase and peroxidases (6,38,54,60). The unusual nature of the thiol redox system in kinetoplastids has been recently established by the discovery of trypanothione (35). Trypanothione [$T(SH)_2$] is a thiol- and spermidine-containing compound with a molecular weight of 721 (36). In contrast to microbes and mammals, where NADPH is the substrate of glutathione reductase, $T(SH)_2$ is an essential redox intermediate for reducing glutathione disulfide (GSSG) to reduced glutathione (GSH) in trypanosomatids (38). Trypanothione reductase (TR) is the rate-limiting enzyme in this reaction (106).

A remarkably similar dependence upon thiols has just been documented in a related kinetoplastid Crithidia fasciculata (108). There is tightly controlled regulation of the transition from $T(SH)_2$ to glutathionyl-spermicine (GSH-SPD) as a major thiol in Crithidia associated with the halting of growth. This is reflected in increased proportion of spermidine bound to GSH as these cells progress from log to stationary phase. When cell growth is restored, the release of spermidine from GSH-SPD in C. fasciculata occurs rapidly (reshuffling is 90% complete within 15 min), and precedes de novo synthesis of GSH or spermidine (108). Hence, this reshuffling has been proposed as a mechanism for providing an immediate source of free SPD for cell division. This has profound implications for drug resistance to DFMO by Leishmania (**Addendum 1, Conclusions**).

There are 3 observations which support the hypothesis that polyamine biosynthesis and glutathione metabolism are linked: 1) $T(SH)_2$ has been found only in other kinetoplastids (Crithidia, Leptomonas, Trypanosoma, Leishmania species), and not in bacteria, mammals, plants, yeasts, or other protozoa (2,35,106) the biosynthesis of $T(SH)_2$ in these genera is absolutely dependent upon the polyamine spermidine (36); and 3) pathogenic kinetoplastids are selectively killed by the polyamine inhibitor, DFMO, and synergistically so when combined with arsenicals known to inhibit thiols (JK, sited in 9; JK & AHF, in preparation; 34).

It is well established both in vitro and in vivo that trypanosome polyamine biosynthesis is irreversibly inhibited by DFMO and a number of putrescine or ornithine analogs (reviewed in 9a and 9b). DFMO, a suicide substrate, inhibits the rate-limiting enzyme ODC by serving as an analog of ornithine and covalently inactivating it. Due to absence of polyamine synthesis, cell division stops, protein synthesis is compromised, and aberrant morphological forms appear (8,27,48).

It is also well known that DFMO, in combination with the antitumor glycopeptide Bleomycin synergistically cures mice of bloodstream and central nervous system trypanosomiasis (9a). When Bleomycin is given to mice treated with DFMO, its uptake is facilitated because of its polyamine (spermidine) sidechains for which trypanosomes are starved. Once inside the cell, Bleomycin binds DNA causing strand breaks, and the cells die (9a).

Glutathione reductase (GR) and other flavin oxidoreductases [TR, lipoamide dehydrogenase (LPH)] are exquisitely sensitive to inhibition by arsenicals which bind an active site histidine (1,2,68,70,90). Incubation of LPH, part of the pyruvate dehydrogenase complex of E. coli, with the substrate and radiolabelled bifunctional p-[(bromoacetyl)amino]phenyl arsenoxide irreversibly inhibits LPH (or GR) activity in a two step reaction. Initially, a stable dithioarsinite complex near the active site is formed, and then, in an exchange reaction alkylation of histidine occurs. Control and experimental subunits isolated from this reaction show LPH activity decreases 81%, and that the only radiolabelled products are N^3-(carboxymethyl) histidine and S-(carboxymethyl)cysteine (1).

Arsenicals also inhibit trypanothione reductase of C. fasciculata and trypanosomes (38). An adduct of dihydrotrypanothione and melarsen oxide (Mel T) has been synthesized. It is an effective in vitro inhibitor of TR from C. fasciculata (K_i = 28 uM). Mel T is the only metabolite rapidly formed in vivo when trypanosomes are treated with MO, and it reaches a maximum intracellular concentration of 20 uM within T. b. brucei (38). This suggests that arsenicals kill kinetoplastids by disrupting intracellular thiol-redox balances and in particular, by irreversibly binding trypanothione (34,38).

It has been presumed that pentavalent antimonials act in a similar fashion to inhibit thiols (13,77). However, our preliminary data for the pentavalent antimonial Pentostam show that it is unable to affect the intracellular thiol-redox balance by irreversibly binding trypanothione (JK and AHF, unpublished). Additional analogs of pentavalent and trivalent anitmonials are currently being tested (see Section C. Antimonials).

These biochemical data suggest that thiols in polyamine and trypanothione metabolism play a unique role in survival of all kinetoplastids, and that the enzymes controlling the reduction of thiols and thiol:disulfide exchange deserve in depth study as new targets for chemotherapy. We also think that studying the genes for the rate-controlling enzymes in these reactions, ODC and TR, will give us excellent clues for devising new methods of control.

b. Results and Discussion

During the last two years of this contract, we have explored these problems. Our main findings are detailed below, and are:

(1. Polyamines, glutathione and trypanothione are present in significant quantities in various Leishmania species. The levels of some of these show significant changes with the life cycle.

(2. DFMO inhibits in vitro growth of some, but not all Leishmania species tested, and the effect can be abrogated by exogeneous putrescine. This inhibition is accompanied by marked depression of trypanothione and polyamine levels.

(3. DFMO is able to suppress infections in laboratory animals. Other drugs acting on the parasite show synergism with DFMO.

(4. Cloning of the genes for the enzymes of polyamine biosynthesis, ornithine decarboxylase (ODC), and of the redox regulation of the cell, trypanothione reductase (TR), is underway. The homology of the first enzyme with its counterpart in T. b. brucei enabled us to use a heterologous probe.

Since much of the data discussed below is in press, that paper is attached as **Addendum 1**, and is extensively referred to for Tables and Figures in the section below.

(1. Polyamines are present at significant levels in promastigotes of three Leishmania species tested extensively by us (L. donovani, L. braziliensis guyanensis, and L. m. mexicana) (68; Table 8, **Addendum 1**). Since our initial in vivo results coincided with the discovery of trypanothine, which is involved in kinetoplastid glutathione metabolism (35), we included the study of this pathway in our program. TR is the rate-controlling enzyme for maintaining significant amounts of reduced glutathione (GSH) in trypanosomatids. All components of the $T(SH)_2$ - GSH system also occur in significant amounts in amastigotes and promastigotes of all Leishmania species tested (68; Tables 6 & 7, **Addendum 1**).

Glutathione is a major thiol [13.7 nmol $(10^8$ cells$)^{-1}$] as in other trypanosomatids. GSH and $T(SH)_2$ are elevated in log phase promastigotes (68; Table 7). As these cells reach stationary phase, GSH remains unchanged, but glutathionyl-spermidine (GSH-SPD) and $T(SH)_2$ levels decrease (68; Table 7, **Addendum 1**). This indicates that regulation of GSH levels is important for these cells during all phases of promastigote development. During both life cycle stages, $_t$rypanothione (2.4 - 3.7 nnmol/min/10^8 cells and 2.6 - 6.2 nmol/min/10^8 cells, respectively) and its reductase activity (Table 6) in Leishmania is high. Each of these is essential for glutathione metabolism and cell survival. These results confirm the importance of thiols in all genera of kinetoplastids tested, i.e. Crithidia, Trypanosoma, and Leishmania.

(2. We have demonstrated that DFMO alone and in combination with other drugs is a specific inhibitor of Leishmania (68; **Addendum 1**). DFMO inhibits in vitro growth and in

vivo replication of L. donovani and L. braziliensis guyanensis, but not L. major or L. mexicana (54). The reason for this difference is unknown. There is no evidence for concentration of DFMO by any of these Leishmania, and minimum inhibitory concentrations of DFMO do not differ significantly among them (0.8 - 2.0 mM; JK, S. Paul, and AHF, in preparation). There is no evidence for alternative pathways or enzymes eg. arginine nor agmatine decarboxylase (JK, SP, & AHF, submitted). Exogenous putrescine (1 mM) reverses growth inhibition by DFMO (**Addendum** 1; Figs. 1 and 2).

As expected, DFMO influences polyamine levels. In both log and stationary phase promastigotes, it reduces putrescine to non-detectable levels, and spermidine and spermine to <50 % of controls. Of special interest is, however, the observation that DFMO also decreases $T(SH)_2$ and GSH-SPD levels in both log and stationary phase promastigotes to 72% and 61% of controls, respectively (**Addendum** 1; Table 7), while GSH levels remain virtually unchanged. Our data are consistent with the hypothesis that polyamine, trypanothione, and glutathione pathways are linked. These DFMO-induced changes in metabolite levels are similar to those reported for Trypanosoma b. brucei (12).

In view of the observations in other trypanosomatids, any compound depleting total GSH or $T(SH)_2$ ought to also be a potent leishmanicide. Recent in vitro data support this view (38). An adduct of trypanothione and melarsen oxide (Mel T) has been synthesized (38). This derivative is the active acid soluble form of the drug formed in vivo when bloodstream T. b. brucei are treated with MO. Mel T also effectively inhibits C. fasciculata TR (K_i = 28 mM).

(3. Our in vivo data confirm the efficacy of DFMO against Leishmania (sited in 9a; 68, **Addendum** 1) infectious in BALB/cByJ mice and inoculated intracardially with promastigotes of L. donovani (MHOM/SD/43/WR 130c), or inoculated intradermally at the shaved base of the tail with promastigotes of L. b. guyanensis (MHOM/SR/81/CUMC 1) or L. mexicana mexicana (MHOM/BZ/58/WR 183).

One percent DFMO combined with Bleomycin (a spermidine-containing, antitumor antibiotic) synergistically suppressed (87%) liver burdens of mice infected with L. donovani (Table 1, **Addendum** 1). DFMO alone suppressed burdens 16%, whereas Bleomycin alone was ineffective. Although the combination of DFMO and Bleomycin did not cure mice of visceral leishmaniasis, inhibition was as effective as by the antimonial Pentostam, since parasites could still be cultured from spleen homogenates after either treatment (Table 1, **Addendum** 1).

Mice infected with L. donovani were also treated with DFMO alone or in combination with a variety of known antikinetoplastid drugs. Without exception, combination of DFMO with Suramin, allopurinol riboside (HPPR), Berenil, or Pentostam improved their

ability to suppress experimental infections in mice (68; Table 2, **Addendum** 1). Suramin alone or in combination was also significantly suppressive. As before, none of these treatments cured mice of their infection.

We have also extensively tested DFMO alone and in combination with Pentostam against experimental infections of L. b. guyanensis and L. m. mexicana (Tables 3,4, **Addendum** 1), as well as L. donovani (Table 5) to see whether DFMO might lower the Pentostam dose necessary to suppress these infections. In each case, >2% DFMO alone in the drinking water was 20-50% suppressive, and against L. braziliensis guyanensis the effect was striking ($p<0.0001$; Table 3). **This is the first time that an oral, specific alternative to antimony has been identified against mucocutaneous disease.**

The effect of DFMO differs in various Leishmania species. DFMO alone significantly suppressed each experimental infection tested (Tables 3-5), but the effect against L. mexicana infections (Table 4) was less than that for L. b. guyanensis or L. donovani (Tables 3,5; **Addendum** 1). As expected, suppression by Pentostam alone was also highly significant against each of these infections ($p<0.0001$, 0.003, and 0.004, respectively). When DFMO was combined with Pentostam, however, the effect was additive at best, and in L. mexicana infections suppression was only marginally better than treatment with DFMO or Pentostam alone. Differences in efficacy upon these infections in vivo may be partly explained by differential drug delivery to tissues, or by a defect in T-cell influx in BALB/c mice infected with L. mexicana (82). They may also be due to species differences in ODC turnover rates, the enzyme's affinity for DFMO, or the regulation of expression of ODC.

Not only is DFMO an inhibitor of these two pathways, but it induces the transformation of cells into a biological deadend by inhibiting mitochondrial enzymes (48). In an elegant study, Feagin and Stuart have shown that DFMO induces stumpy bloodforms of trypanosomes, and that the initiation of this event can be detected in kinetoplast (mitochondrial) transcripts prior to the morphological change (39,41).

The importance of these two pathways for Leishmania has been established by us, and has resulted in a proposal for Phase 1 clinical testing of DFMO in combination with several compounds against leishmaniasis in the Americas. Phase 1 clinical trials against African trypanosomiasis are already in place in Kenya (103). These data suggest that DFMO, or more powerful ornithine analogs, in combination with known antileishmanial agents could provide alternatives to antimonials. These might be less toxic and more specific against all forms of the disease.

Currently, we are testing several new ornithine analogs against VL, CL, and MCL in our animal models. Lipophilic ester derivatives, eg. 3,4 dehydro monofluoromethylornithine

methylester ($MFMO.CH_3$) have proved 4x more effective than DFMO against experimental trypanosomiasis (9,80), and are believed to act as prodrugs which can be hydrolyzed by the cell to active forms (80). $MFMO.CH_3$ was also 5x more active in curing experimental CNS trypanosomiasis than DFMO if it was combined with a single dose (20mg/kg) Suramin (8). Since our data (see **Addendum 1**) show an additive effect against experimental VL of DFMO with Suramin (Table 2), it is likely that $MFMO.CH_3$ plus Suramin will also be several times more potent gainst leishmaniases than DFMO in this animal model.

There are, however, naturally-occurring species of Trypanosoma, and experimental mutants of Leishmania resistant to DFMO. In the former, some African strains require 6-8x more DFMO to eliminate experimental infections from the bloodstream of mice (C. Bacchi, pers. comm.). This is not due to gene amplification (12), but appears to be due partially to alternate routes for the generation of excess ornithine, putrescine and spermidine during polyamine biosynthesis. Mutants produced experimentally in vitro have been reported to have altered permeability to the drug (94), but others have been unable to substantiate this (12). The selective toxicity of DFMO for trypanosomes has been shown to be partly due to slower turnover rate of ornithine decarboxylase (ODC) the rate limiting enzyme in polyamine biosynthesis than in mammalian cells (93). This allows the drug a greater opportunity to irreversibly bind ODC. The difference in turnover rate is due to the addition of 20 nucleotides (nt) at the amino terminus of the protein, and a deletion of 36 nt at the carboxy end (see section 2 for details).

It has also been shown that these strains of trypanosomes treated with DFMO have higher levels of S-adenosylmethionine (SAM), SAM decarboxylase, and other intermediates in the methylation process (137). Although the significance of this finding is not yet known, it has been prompted an investigation of the rate-limiting enzyme in this pathway, SAM synthetase, and the methylation pathways in resistant strains of trypanosomes (C. Bacchi). We are examining these pathways in Leishmania.

Based upon these rather remarkable and new findings, it seemed logical and even imperative to us, to attempt understand these processes at the molecular level. As a result of our preliminary biochemical data, we initiated a study one year ago to isolate and clone the ODC and TR genes from Leishmania.

2. Molecular Aspects

a. ODC Genes - Background

Until the last few years, virtually nothing was known about ODC genes in lower eukaryotic cells, whereas mouse ODC had been cloned, characterized and sequenced (55,81). Mouse ODC is a 51,172 M_r protein containing 461 amino acids arranged in alternating domains of alpha helices and beta sheets (81). It has

no known sequence homologies with other proteins, and is rapidly turned over. Southern blot analysis of wild type and mutant genomic DNAs suggest most mammalian ODCs belong to a multigene family (3,4,81).

In 1987 using mouse cDNA as a probe, and under stringent conditions of hybridization, the gene encoding ODC in Trypanosoma b. brucei was cloned and sequenced (93). This gene encodes a protein of 445 amino acids which is 62% homologous to mouse ODC (81). In contrast to mouse ODC, however, the gene in T. b. brucei does not belong to a multigene family (72), and it has a slow turnover rate (93). This is due to a 20 amino acid addition and 36 amino acid deletion at the N- and C-termini, respectively, and may account for the selective toxicity of DFMO for trypanosomatids (9a). The ODC gene of trypanosomes does not have consensus sequences found in other trypanosomatid genes (83,92), nor eukaryotic genetic consensus elements (93). Like other kinetoplastid genes, there are no introns, and the gene is G+C rich, with a codon bias in the third position (66,93). This bias explains the difference in restriction maps between mouse and trypanosome ODC, in spite of >60% DNA homology.

Unlike T. b. brucei, the ODC gene recently cloned from the yeast Saccharomyces cerevisiae shares little sequence homology with mouse ODC (43). In view of the evolutionary distance between trypanosomatids and mammals, and the lack of similarity between yeast and mammalian ODC, it has been suggested that T. b. brucei may have acquired part of its host genome for ODC (93). However, comparison with other eukaryotes indicates that the active site sequence, and the secondary structure of ODC are highly conserved (62), and that sequence differences occur chiefly in the 5' and 3' flanking regions of ODC. These are probably unique for yeasts because they are essential for cell cycle regulation and mating type switching (43).

There is no evidence in T. b. brucei for gene amplification of ODC in response to DFMO (12,94). Yet this is a common mechanism of DFMO resistance in mouse and human cell lines (3,74). In these cells, only one of the genes in the multigene complex is amplified in response to DFMO, and the gene product is altered (3) indicating that several mechanisms of resistance may occur. This is consistent with recent reports that DFMO-resistant T. b. brucei exhibit altered drug uptake, increased ornithine synthesis, and possible efflux of DFMO (12,94).

In mammalian cell lines, methylation polymorphisms also influence amplification of the ODC gene, overproduction of the enzyme, and increased transcript abundance (3). It is even speculated that capacity for metastasis of some tumor cell lines is related to these polymorphisms (4). In trypanosomes, DFMO induces a change in the abundance of several mt maxicircle transcripts, and we have preliminary evidence that methylation polymorphisms occur in the mt DNA of virulent and avirulent clones of L. b. guyanensis (JK and C. Leonard, unpublished).

Whether this is related to the faster growth rate of one of the clones, and/or its ability to cause metastatic mucocutaneous disease in experimental animals is as yet unknown. It will be interesting to determine whether methylations occur in the ODC nuclear genes of Leishmania, and if so, whether methylation polymorphisms in either mt DNA or nuclear DNA can be related to virulence, pathogenesis or drug resistance. It will also be interesting to see whether the biochemical methylation index of the cells, as measured by SAM synthetase expression, can be related to DFMO resistance (137).

ODC is a low abundance cytosolic protein in most eukaryotic cells (0.01 - 0.05%), and is occasionally expressed in nucleoli (117). The enzyme is differentially expressed during the life cycle of yeasts (Saccharomyces), slime molds (Physarum), and fungi (Aspergillus, Neurospora) (44,117). In these cells, bacteria and mammals, enzyme induction can occur by treatment with androgens, serum, nerve and platelet growth factors, and by polyamine depletion when cells are placed in fresh medium (3,58). Specific activities of the enzyme vary, but in general they are lower in prokaryotes and protozoa (0.1 - 33 nmol mg^{-1} hr^{-1}), than in slime molds, fungi, or yeasts (9 nmol - 31 umol mg^{-1} hr^{-1}), and mammalian cells (30 umol mg^{-1} hr^{-1}) (49,117,136).

b. TR Genes

Similarly to ODC, until very recently, little was known about the genes for flavoprotein enzymes, especially the disulfide oxidoreductases which include GR, LPH, mercuric reductase (MR), thioredoxin reductase, and TR. However, within the last 5 years, the genes for E. coli GR and LPH, as well as Pseudomonas aeruginosa MR (carried by transposon 501) have been cloned and sequenced (18,53,115). These genes encode flavoenzymes of ~450-550 amino acids with an M_r of ~55,000. The precise location of human GR has been identified on chromosome 8 (63a). The amino acid sequence of all these flavoenzymes is remarkably conserved (>65%), and with the exception of thioredoxin reductase, indicates evolution from a common ancestor (90). There is even a similar G+C bias in the third codon position.

Partial sequence data for C. fasciculata TR shows this enzyme has >85% homology with human GR (71,106). We have used this information to construct several mixed oligonucleotides complementary to the amino acid sequence of Crithidia TR, and to prime reverse transcription of total RNA from several kinetoplastids (detailed in Preliminary Results). Except for strict anaerobes and some archebacteria, GR is present in meaningful amounts in all eukaryotes and prokaryotes (90a), and has been isolated and purified from a variety of them including cyanobacteria, human erythrocytes, and protozoa (71,105,106).

These molecular data indicate that the genes for ODC and GR have retained much of their structural and functional properties during evolution from primitive to more advanced life forms.

However, these genes have also evolved and retained unusual properties associated with the organisms mode of life. In view of the important role ODC and TR play in the survival of trypanosomatids, we think that an examination of the genes of species of Leishmania and their expression will provide clues for the development of new and better drugs, as well as an understanding of how resistance develops.

c. Results and Discussion

As mentioned, until recently very little was known about the genes for ODC in lower eukaryotic cells. Within the last year, however, the complete sequence of ODC from Trypanosoma brucei brucei was published (93), and we were graciously provided (by C.C. Wang, UCSF) with a 2.2 kb SstII - HincII ODC fragment cloned into the Bluescript vector (Stratagene). The plasmid with its insert was amplified by us in DH5 alpha E. coli, and its identity confirmed by gel electrophoresis using uncut and HincII-cut Bluescribe (Stratagene), total plasmid DNA digested with SstII and HincII, and HindIII:EcoRI lambda as markers. An insert of appropriate size was observed.

Once its identity was established, we used alpha ^{32}p-labelled ODC to probe L. b. guyanensis DNA by Southern blot analysis (112). DNA was extracted from stationary phase promastigotes by established methods (56), and was digested with BamHI, EcoRI, PstI, SstI, and PvuII. Restriction fragments were electrophoresed in 0.7% agarose gels and transferred onto Nytran (Schleicher & Schuell). Using conditions of high stringency (60^{o}C, 0.1xSSPE) hybridization of the T. b. brucei ODC probe to L. b. guyanensis DNA shows a major band on SstI digests at 5.5 Kb.

These data indicate that we may have identified the Leishmania ODC gene and that, as in trypanosomes, it is probably a single gene rather than part of a multigene family as in humans and mice (81,94). These results also tenatively confirm the validity of this probe for future use in identifying ODC in northern and pulsed field gel electrophoresis (PFG) blots of chromosomes (47,104), and for screening cDNA and gDNA libraries of Leishmania as well. Absolute confirmation awaits sequencing the Leishmania gene, and comparing it with that of trypanosome, yeast, and mammalian ODC (43,55,94).

As discussed, the amino acid sequence for Crithidia fasciculata TR is partially known (106), and shows >85% homology with human GR (71). We have used this information to construct a synthetic, mixed oligonucleotide (11 nt) near the N-terminus. The rationale for selecting this region initially was based upon its G+C rich properties, its potential for priming, and its specificity (the C-terminus contains the active site sequence which is highly conserved and likely to cross-react with other flavin oxidoreductases in the cell eg. GR, LPH (53).

The primer was end-labelled with gamma ^{32}P-ATP, and its size checked by analysis on a 20% sequencing gel using lambda linkers varying in size from 6 to 13 nt. Alpha ^{32}P oligomer was hybridized under stringency conditions determined empirically for a mixed oligomer of 11 nt (35° C, 0.1X SSPE) to Southern blots of L. b. guyanensis described above. Because of its small size and degeneracy, hybridization was less efficient than that for our heterologous ODC probe. Although we were able to see several bands of hybridization in BamHI and EcoRI digests at approximately 11.5 - 12.5 kb; in PstI at 2.2 and 2.6 kb; in SstI at 3.5, 4.4, 5.0, 6.0, 8.0, and 11.5 kb (data not shown), we thought these data too uncertain to use this probe for further hybridizations or for screening libraries. However, they were encouraging, in that they confirmed the ability of a small mixed oligonucleotide based upon the amino acid sequence of C. fasciculata TR to see DNA in another kinetoplastid, L. b. guyanensis.

In order to construct a more specific probe, 150 - 350 ng of labelled primer was annealed to 5 ug of total RNA from homologous C. fasciculata, and heterologous T. b. brucei, or L. b. guyanensis in a reaction mixture (10.5 uL) containing 40 or 80 mM KCL (pH 8.0) and was incubated at 42° C for 5 min, 37° C for 40 min, and 27° C for 15 min. Following hybridization, the reactions were centrifuged briefly, and 12 uL of buffer mix [MMLV RTase buffer, a ^{32}P ATP, H_2O, MMLVRtase (BRL, Bethesda, MD)] was added. For sequencing, 4 uL of each reaction was aliquoted into each of 5 tubes containing 1 uL each of appropriate nt mix (CATGX). Nt mixes contained 10 mM Tris buffer (pH7.4), 10 mM dNTPs, 1 mM dNTP, 5 mM ddNTPs, and H_2O. (X lanes were extensions only, and are included as an internal control) An additional control with just RNA and no primer was included. Extensions were incubated at 37° C for 30 min, and chased with dNTPs for 15 min at 37° C. Extensions were brought to room temperature, and formamide/sequencing dye added prior to heating in an oil bath at 60° C. Products of MMLV reverse transcription were sequenced using a 12% polyacrylamide denaturing gel.

For primer extension and sequencing, gamma ^{32}P labelled 11- and 26-mixed oligonucleotides of TR, and L. tarentolae cyt B or T. b. brucei cyt B extension products served as size markers and controls. Interestingly, using the TR 11 nt oligomer, extension products were only observed using C. fasciculata and L. b. guyanensis RNA, and not T. b. brucei RNA. These results may indicate significant differences in the TR from kinetoplastid genera. There are several well-labelled extension products in C. fasciculata between nt 85 - 100 and one major 75-85 nt extension product in L. b. guyanensis. Because I obtained more reliable results with homologous C. fasciculata RNA during primer extension, this species was used in an attempt to sequence of TR, and to obtain a longer, more specific TR probe for Southern, northern, and PFG hybridizations.

The results shown below indicate that the starting sequence determined for C. fasciculata is consistent with the authentic sequence predicted from the known amino acid sequence of TR (82). There is one unknown amino acid at the predicted N-terminus (XXX). The first seven nucleotides which can be read begin at the predicted 3' extension of the oligomer, and there is an AUG start site in frame approximately 9 nt 5' to the N-terminus of TR (|). Within the first 25 nucleotides which can be read with certainty, there are 7 which are an exact match (*). The sequence has been determined for 123 nt 5' from the active site.

C. fasciculata RNA sequence (5' > 3')

```
          45      - -             - ---      11
Observed    XGCATGGCXCGCXAXXXCCXXCATXTCTCCCCXCX
Predicted                   XXXCAACAACGXCCXCCXCA
              |||             *  **     * ** *
```

* match of observed to predicted
X ambiguous , - questioned, | start

We have examined these data to see whether we have reached the 5' end of the transcript by searching for the 35 spliced leader sequence of L. enriettii, L. collosum, T. cruzi, and T. b. brucei (83,92). Evidence suggests we have not. In addition to these data, we have constructed and are testing 6 additional probes varying in length from 17 to 41 nt. These are based upon recently determined codon usage in Leishmania, and sequences for the redox active site of falvin oxidoreductases over a wide range of organisms.

B. Imidazoles (Allopurinol, Allopurinol Riboside)

The selection of allopurinol as a potential new drug for the treatment of leishmaniasis is based upon studies of the enzymology and biochemical pathways of purine and nucleic acid metabolism in leishmania and in mammalian cells (7,75,77,113). It is widely employed for the treatment of gout and other hyperuricemic conditions in man (13). The formation of nucleotides in Leishmania depends to a large extent on the phosphorylation of nucleosides or bases of host tissue origin (75,77). The analog of hypoxanthine, allopurinol, effectively inhibits this process. In uncontrolled human trials, the drug used alone may be effective in the treatment of visceral (64,121), but not mucocutaneous leishmaniasis (78). Allopurinol in combination with Pentostam cured five patients with visceral leishmaniasis who were unresponseive to Pentostam alone (23).

More recently, allopurinol and its riboside have been used in combination with other drugs against leishmaniasis (13,77,121). Our own data suggest that allopurinol and its riboside, and the related compound 9-deazainosine in combination with DFMO have enhanced effects against visceral leishmaniasis (see Results; 68). Our collaborative work with R.L. Berens and

J.J. Marr (U. Colorado) and R. Klein (Sloan-Kettering Cancer Res. Inst.) has determined that a novel purine analog, 9-deazainosine (9-DINO), has a synergistic effect when combined with 3% DFMO against experimental VL (see Results). This drug also cures >90% CNS infections of African trypanosomiasis in mice, is apparently non-toxic, and is converted to toxic metabolites only by the kinetoplastids and not the host (9b). Thiol analogs of this drug are currently being tested against both trypanosomiasis (CB) and leishmaniasis (JK).

C. Azoles (Ketoconazole)

Recently oral anti-leishmania drugs other than allopurinol have also been tested in humans. Azoles (itraconazole, ketoconazole, fluzonazole) and allylamines have been identified as promising leishmanicidal compounds (17,51,65,101). Terbinafine is the most effective compound so far tested in the new class of allylamine antimicrobial agents. It has been shown to inhibit the enzyme squalene epoxidase, a key enzyme in ergosterol biosynthesis (101). Its activity is highly selective, and is more inhibitory to fungi (or leishmania) than to mammals. The reason for this is the action of these drugs upon the unique biosynthetic pathways for ergosterol in Leishmania (50).

As before, these data are in press. The paper is included as **Addendum 2**, and is extensively referred to for Figures.

1. Results and Discussion

We have recently shown (**Addendum 2**;52) that the major sterol identified in the promastigotes of several Leishmania species is one of a family of ergosterols (50). The presence of lanosterol and other trace sterols in Leishmania (52) as measured by 2-^{14}C mevalonic acid indicate that the major promastigote sterols are produced by metabolic routes similar to those of fungi (122). A range of imidazole and triazole antifungal drugs have been designed which block the chtochrome P-450 dependent C-14 demethylation step in ergosterol synthesis of fungi (123). This causes the accumulation of 14 alpha-methyl compounds (eg. lanesterol) and a decline of ergosterol (122). This changes the composition of the plasma membrane and its enzyme functions which, in part are the basis of fungistatic and fungicidal effects of azoles (123).

The similarity of sterol patterns between species of Leishmania and fungi prompted us to test the effect of ketoconazole against L. m. mexicana in vitro (**Addendum 2**; 52). Growth of both promastigotes and amastigotes was inhibited, and as expected, 14 alpha methyl sterols accumulated with a concommitant decrease in C_{28}-sterols. Since amastigotes of Leishmania reside within macrophages which have the ability to synthesize cholesterol, we decided to determine the sterol content of amastigotes to be sure that the sterols present were not derived from host macrophages. The uptake and utilization of

host sterols by amastigotes would have important implications for the successful use of imidazole drugs, or other sterol inhibitors for use against leishmania.

Promastigotes of L. braziliensis guyanensis and L. m. mexicana contain cholesterol, derived from the medium, and synthesize 5,7-C_{28}-sterols. However, the ergosterol products derived from these syntheses are quite different between the two species. There is no evidence of $5C_{28}$ sterols in promastigotes (**Addendum 2**).

Amastigotes of both species, on the other hand, contain cholesterol and desmosterol which are products of the host macrophage (52; **Addendum 2**). The amounts of 5,7-C_{28}-sterols are decreased but C_{29} sterols appear and account for about 20% of sterols. In addition, 5-C_{28}-sterols do appear in amastigotes. Both of these species of Leishmania produce ergosterols, but there are two additional members of the family produced by L. braziliensis guyanensis. These differences may account for the difference in efficacy of antifungal imidazoles for these two species (17,26,122,126,134), and has profound implications for chemotherapy of the leishmaniases. As reported below, the efficacy of azoles for cutaneous and mucocutaneous trials in patients is quite variable.

Ketoconazole has been previously tested by us for activity in vivo against Leishmania species, without remarkable effects (Annual Report 2, DAMD17-C-0061). However, others have shown this azole inhibits L. m. mexicana promastigotes (14,51) and amastigotes within human and mouse macrophages in vitro (13,96,122). This drug is also effective against VL in hamsters (11), and to some degree against cutaneous and mucocutaneous infections in humans (13,96,122).

The use of azoles against leishmaniasis is still controversial. Oral ketoconazole has been used at high doses once or twice daily for 3 months to treat human cutaneous and mucocutaneous leishmaniasis (122,134). Clinical improvement was remarkable, although some patients experienced somnolence and dizziness (122). However, neither the treatment regime nor Therapeutic Index was competitive with Pentostam. Since our last report, additional failures have been documented against L. braziliensis guyanensis (26) and L. b. braziliensis (78), while moderate success has been seen against Old and New World cutaneous infections of long-standing (126).

Within the last three months, we have had experience with a 9 year old patient from Afghanistan who presented with two lesions on his forehead and nose. Biopsy of the former lesion revealed infection with L. tropica, as tested by monoclonal antibodies and isoenzyme typing. Because of his age, the physicians were reluctant to treat with antimonials, and elected to treat with ketoconazole. Clinical improvement of the forehead lesion was remarkable, but the nasal lesion did not resolve (T.

Funt, M.D., pers. comm.). Because ketoconazole did not cure this patient, he is now being treated with a course of Pentostam.

2. Problems

The lack of correlation of in vitro with in vivo data on the use of azoles against leishmaniasis, indicate that these drugs will remain controversial. It appears that most azoles are leishmanistatic, rather than leishmanicidal, and that combination chemotherapy will be necessary for routine use in patients. As in polyamine and thiol inhibitors newly tested, species of Leishmania exhibit remarkably different sensitivities to most groups of drugs. In the past, this aspect of chemotherapy has not been fully appreciated. It appears that much more needs to be known about the biochemical and molecular basis of these species-specific differences before CL, MCL, and VL can be cured by chemotherapy. A "magic bullet" for all species of Leishmania is an unlikely prospect.

C. Antimonials.

Although therapy for all forms of leishmaniasis is unsatisfactory (20,31,79,90b,99,100,127,129,130), systemic pentavalent antimonials remain the treatment of choice for all leishmaniases. Recently, longer regimes and higher doses have improved cures and decreased relapse rates in humans (19,21,22,120). Combination of Pentostam with rifampicin or allopurinol has improved therapy of resistant cutaneous and visceral leishmaniasis (23,91a). The latter drug combination has been recommended for study by the WHO Special Program for Research and Training in Tropical Diseases.

We had previously reported the potentiation of Pentostam using BCG (Ann. Report 2, DAMD17-80-C-0016). Previous studies using BCG against cutaneous leishmaniasis were inconclusive. Mice pretreated with BCG were better able to control L. tropica infections, as measured by reduction of lesion size and metastasis to viscera (134a), but BCG was unable to alter either the course of infection or the immunological response of C3H mice to infection with L. mexicana (54a). Neither BCG nor levamisole alone altered L. mexicana infections.

More recently others have reported the potentiation of Pentostam with interferon gamma (IFN-gamma;87). Other forms of immunotherapy currently being evaluated in the treatment of leishmaniasis alone and in combination with Pentostam or Glucantime are granulocyte monocyte-colony stimulating factor (GM-CSF), interleukin 2 (IL-2), IL-1, and macrophage colony stimulating factor (M-CSF). All of these are available in recombinant form (eg. rIFN-gamma, rGM-CSF), and have been previously evaluated in the treatment of humans (88,98,135).

The mode of action of Pentostam is still unknown. Since our last report, however, several studies have attempted to elucidate

its action *in vitro* against amastigotes in macrophages or promastigotes in culture (13). Although absolute amounts of antimony in animal tissues have been measured (99), nothing is known about its active metabolites. We do know that antimony accumulates within the liver and spleen (89a), and that ^{125}Sb sodium stibogluconate accumultes within amastigotes *in vitro* (25). However, previous suggestions that the mechanism of action of antimonials was binding glycolytic enzyme -SH groups (86) has not been substantiated (34). In addition, our preliminary evidence indicates that the unique Leishmania SH-containing target, trypanothione [$T(SH)_2$], is not a target for inhibition by pentavalent antimony (JK and A. Fairlamb, in preparation).

Recent evidence in our laboratory, however, suggests that species of *Leishmania* consistently show differences in susceptibility to a wide range of drugs (68). These differences can be documented both *in vivo* and *in vitro*, and suggest a genetic basis for resistance. As mentioned before, at the molecular level, some of these species are able to produce higher levels of enzymes by gene amplification necessary to maintain metabolism in the presence of the drug, some alter membrane permeability to drug, and others express proteins which have altered turnover rates of enzyme (93).

We are currently testing the ability of *Leishmania* species to express enzymes of the polyamine and trypanothione pathways while under antimony pressure. Pentostam- and Triostam-resistant promastigotes are being obtained stepwise essentially as described for methotrexate-resistant *L. major* (15). Amastigotes are being obtained from BALB/c J774.1N- macrophages using techniques previously described by us (24). Drug-treated and untreated macrophages with and without amastigotes are being studied biochemically, and these will be used for our studies. They are being compared with axenic amastigotes of *L. mexicana pifanoi* in collaboration with Dr. P. Rainey (Yale University).

Analysis of the major metabolites of trypanothione formed *in vivo* after treatment with Pentostam or Triostam will employ techniques used routinely by us (68). Purified trypanothione and its adducts will be used as standards (38). Adducts of $T(SH)_2$ with antimonials are being prepared in collaboration with Dr. O. Griffith (CUMC), following the procedure of Fairlamb, Henderson, and Cerami (pers. commun.). It is expected that adducts of trypanothione with one or more derivatives of antimony can be formed, since the reactive sites and solubility in aqueous solution are similar to those of the arsenical melarsen oxide previously measured (34,38). Amino acid and mass spectrometric analysis for the presence of antimony derivatives will be performed by Dr. Rainey.

In this way, we hope to eventually understand the mode of action of Pentostam, and other antimony derivatives in order to develop new compounds specifically for each species of *Leishmania*. We think it is imperative to determine the major

target or targets for antimonial drugs, and to understand how resistance develops at the biochemical and molecular level. We do not think any of the data to date have approached this problem in leishmaniasis.

IV. New Direction: Virulence of Leishmania braziliensis. Implications for chemotherapy.

Since the last report, we have identified an unusual RNA virus within the cytoplasm of several species of Leishmania braziliensis (**Addendum** 3; 116,119). In view of the differences in virulence seen among and between clones of L. b. guyanensis (JSK et al, in preparation), and the severity of mucosal damage caused by L. b. braziliensis we have begun characterizing this RNA virus (116,119). The implications of its presence in those species of Leishmania causing mucocutaneous disease, and its implications for future chemotherapy and vaccine development cannot be minimized.

The presence of viruses in parasitic protozoa may be relevant to the resulting diseases and useful for molecular biological studies. DNA viruses have been found in Amoeba (30), double stranded RNA viruses have been found in Giardia (132) and Trichomonas (133), and circular DNAs have been detected in Leishmania (15,45). Additional uncharacterized virus-like particles have been observed in Kinetoplastida (84,85).

We examined 12 stocks of New World and one stock of Old World Leishmania species, and found two that contain an abundant 6000 nt nucleic acid (**Addendum** 3; Table 1, Fig.1). This nucleic acid was absent from Trypanosoma brucei and Trypanosoma cruzi. The high fluorescent intensity for the size of the nucleic acid from L. braziliensis guyanensis CUMC1 1-A in ethidium bromide stained gels indicates that it has a high copy number. It is degraded by RNAse and alkali, but not by DNAse (Fig. 2A) indicating that it is an RNA virus. It has been designated LR1 (Leishmania RNA 1). The sensitivity to RNAse was retained throughout salt concentrations up to 200mM, indicating that the RNA is single stranded. RNA preparations reveal it has a density greater than 1.7 g/cm^3 which indicate again its single-strandedness (131). A similar sized nucleic acid, designated LR2 has been found in L. b. braziliensis CUMC 3, but its relationship to LR1 is unknown. LR1 is retained following passage of L. b. guyanensis CUMC1 1-A through the sandfly (JSK, M. Warshofsky, E. Rowton, and P. Lawyer, in preparation). A segment of LR1 RNA was cloned as a cDNA from gel-purified 6000 nucleotide RNA, and thus is probably from the viral genomic RNA rather than a transcript. The 5-30 cDNA clone hybridized to RNA from L. b. guyanensis CUMC1 1-A, but not to RNA from L. b. braziliensis M2903 or L. major Jericho II, nor to T. brucei RNA (Fig. 1B). Thus, the 5-30 cDNA clone originates from LR1 and has a restricted distribution among species and subspecies of kinetoplastids.

LR1 RNA is largely, if not exclusively, located in the cytoplasm. The cDNA clone 5-30 hybridizes strongly in northern blots to 6000 nucleotide RNA from the cytoplasmic fraction (Fig. 3). A smear of hybridization corresponding to 2000 and 2300 nt RNAs is also seen. There is no hybridizaton to RNA from the nuclear fraction in the experiment shown, however, sometimes slight hybridization to nuclear RNA was detected, probably due to cytoplasmic contamination since the nuclear fractions were not washed. Identical results are obtained whether the cells are ruptured using Triton-X100 or silica carbide grinding (114). Since the nuclear fraction also contains membrane components, these experiments indicate that little, if any, LR1 is associated with the cell membrane. The 5-30 cDNA did not hybridize to Southern blots of genomic DNA from L. b. guyanensis CUMC1 1-A. These experiments incorporated sensitivity controls detecting less than one copy per genome equivalent of the cDNA sequence. Thus, there is no genomic DNA copy of LR1 indicating that it is not a retrovirus.

When the SDS/proteinase K treatment of the cytoplasmic fraction was omitted, a smear of hybridization extending from the well to about the 9000 nucleotide region of the gel was observed (Fig. 3). This suggests that LR1 is a complexed form, perhaps as a particle. This was further examined using sucrose density gradients. Most 6000 nt LR1 RNA is found in the fraction corresponding to a sedimentation coefficient of 130S (Fig. 4). There is also 6000 nt LR1 RNA in the fraction corresponding to 40S, which may be free RNA. Electron microscopic analysis of the 130S fraction revealed spherical particles with a diameter of about 32 nm (Fig. 5). These particles were not present in comparable sucrose density gradient fractions from L. b. guyanensis M4147, the WHO type specimen, which lacks the LR1 RNA. The coincidence of the LR1 RNA and the 32 nm particles in the same fraction, and their apparent absence in cells lacking LR1 RNA suggest that the particles contain the LR1 RNA.

We have concluded that LR1 is an RNA virus. The size and morphology of the particle, the size of the RNA, its maintenance during fly transmission (JK, et al, in preparation), and its cytoplasmic location are consistent with the properties of other RNA viruses. Interestingly, the virus-like particles seen in L. hertigi (84) are also restricted to the cytoplasm. Infectivity experiments with LR1 are in progress. Nucleotide sequence analyses, reveal that the 5-30 cDNA clone from LR1 encodes a protein that is homologous to the L protein of vesicular stomatitis virus. THe L protein is a subunit of the RNA-dependent RNA poly erase of VSV and is conserved among RNA viruses (85).

The effects of LR1 on the parasite are not obvious. There is as yet no correlation between the presence of LR1 and any disease characteristic, host range, or growth characteristic of L. b. guyanensis. The origin of the virus is also unknown, although it is intriguing that the sandfly vector for Leishmania,

is also a known vector of RNA viruses. The initial characterization is insufficient to determine whether LR1 belongs to an existing class or novel class of RNA virus. More importantly, LR1 is a potential transformation vector for Leishmania, and may be a way in which genetic information could be targetted into the cytoplasm of these organisms in order to disrupt their life cycle. It also suggests that, for chemotherapy of mucocutaneous disease, antiviral agents in combination with other drugs should be explored. **This opens a new direction for the chemotherapy of leishmaniasis.**

V. References

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ADDENDUM 1

INHIBITION OF LEISHMANIA SPECIES BY α-DIFLUOROMETHYLORNITHINE

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INTRODUCTION

DL-α-difluoromethylornithine (DFMO) is a specific, irreversible inhibitor of ornithine decarboxylase (ODC), the rate-limiting enzyme in trypanosome polyamine biosynthesis (reviewed in 1). DFMO cures experimental infections of African trypanosomiasis, and is in phase 1 clinical trials[2]. Although the mechanism of selective toxicity is incompletely known, DFMO has well known effects upon the metabolism and morphology of trypanosomes[1]. Following depletion of the polyamines, putrescine and spermidine, by DFMO, long slender trypomastigotes change into stumpy-like forms which are eliminated by the host. DFMO alters mitochondrial transcripts in these cells[3], and reduces their trypanothione [$T(SH)_2$] levels[4]. As reviewed elsewhere in this volume (Fairlamb, A.H.), $T(SH)_2$ has two important roles in kinetoplastid metabolism: the maintenance of intracellular redox balance and protection against toxic oxygen derivatives and other radicals. The selective toxicity of DFMO for ODC in trypanosomes has also been attributed to this enzyme's slower turnover rate than in mammalian cells[5].

Recently, DFMO was shown to inhibit growth of Leishmania donovani promastigotes[6] Here we report its inhibitory effect upon 3 additional species of Leishmania, and extend these data to include suppression of experimental infections in mice.

MATERIALS AND METHODS

Drug Testing in vivo

All experiments were performed in triplicate. P values compare efficacy of treatment with control (untreated, infected) mice. Data were analyzed by the 2-way analysis of variance (Tables 1,2), and by the Neuman-Keuls multiple range and Kruskal-Wallis tests (Tables 3-5). In the latter, significance measures the efficacy of treatment of all dose groups combined, eg. 0.5 through 5.0% DFMO alone, with that of controls.

Additive Effect. Mice infected with L. donovani were also treated with DFMO alone or in combination with a variety of known antikinetoplastid drugs. Without exception, combination of DFMO with Suramin, allopurinol riboside (HPPR), Berenil, or Pentostam improved their ability to suppress experimental infections in mice (Table 2). This was true whether drugs were given before (Table 2) or at time of infection (data not shown). Suramin alone or in combination was also significantly suppressive. As before, none of these treatments cured mice of their infection.

Effect of DFMO upon Cutaneous Infections

We have also extensively tested DFMO alone and in combination with Pentostam against experimental infections of L. b. guyanensis and L. m. mexicana (Tables 3,4), as well as L. donovani (Table 5), to see whether DFMO might lower the Pentostam dose necessary to suppress these infections. In each case, >2% DFMO alone in the drinking water was 20-50% suppressive, and against L. braziliensis guyanensis the effect was striking ($p<0.0001$; Table 3). This is the first time that an oral, non-toxic alternative to antimony has been identified against mucocutaneous disease.

Table 2. Additive Effect of DFMO and Other Drugs upon Leishmania donovani

Treatment*	Dose mg/kg/day	Liver Burden Mean + SD	Percent Suppressn.	Signif.	Cultures +/Total
Saline alone	---	509 ± 342	0	---	2/2
DFMO alone	---	239 ± 77	53 + 15	---	2/2
Suramin alone	20	151 ± 68	71 + 13	p <0.01	2/2
Suramin + DFMO	20	108 ± 66	79 + 13	p <0.01	2/2
HPPR alone	200	229 ± 214	55 + 44	---	2/2
HPPR + DFMO	200	133 ± 100	74 + 20	p <0.05	2/2
Berenil alone	20	160 ± 70	69 + 14	---	2/2
Berenil + DFMO	20	103 ± 25	80 + 5	p <0.05	2/2
Pentostam alone	20	267 ± 189	48 + 37	---	2/2
Pentostam + DFMO	20	115 ± 105	78 + 21	p <0.01	2/2

*DFMO = 3%; other drugs = SC. All started 7 days before infection.

Table 3. Effect of DFMO and Pentostam upon Lesion Size* of Leishmania braziliensis guyanensis.

DFMO	Pentostam (mg/kg/day)				
(%)	0	6.25	12.5	25	50
0	5.3 ± 3.0	7.8 ± 2.0	6.0 ± 0.9	2.2 ± 2.9	2.1 ± 2.7
0.5	8.3 ± 0.6	8.1 ± 1.8	8.2 ± 1.6	7.7 ± 1.5	8.2 ± 1.4
1.0	0.7 ± 0.6	3.7 ± 5.5	2.9 ± 4.0	3.1 ± 3.6	4.4 ± 3.4
2.0	0.0	7.2 ± 0.3	6.6 ± 0.5	6.2 ± 1.0	0.2 ± 0.3
4.0	3.0 ± 2.0	6.9	9.4 ± 2.6	3.9 ± 2.6	3.8 ± 2.5
5.0	1.0	5.7 ± 1.2	6.4 ± 0.8	6.0 ± 0.4	0.0

Significance: DFMO alone p <0.0001
Pentostam alone p <0.0001
DFMO + Pentostam p <0.019

*Diameter in mm

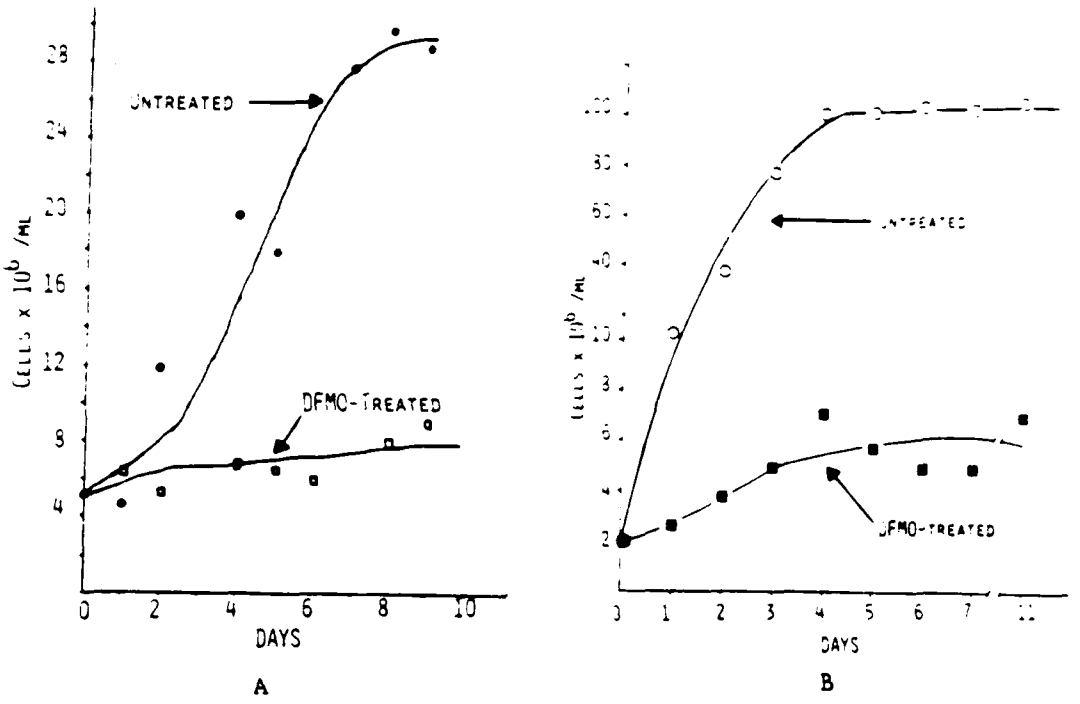

Fig. 1. Effect of 5 mM DFMO upon the growth of L. donovani (A) and L. braziliensis guyanensis (B).

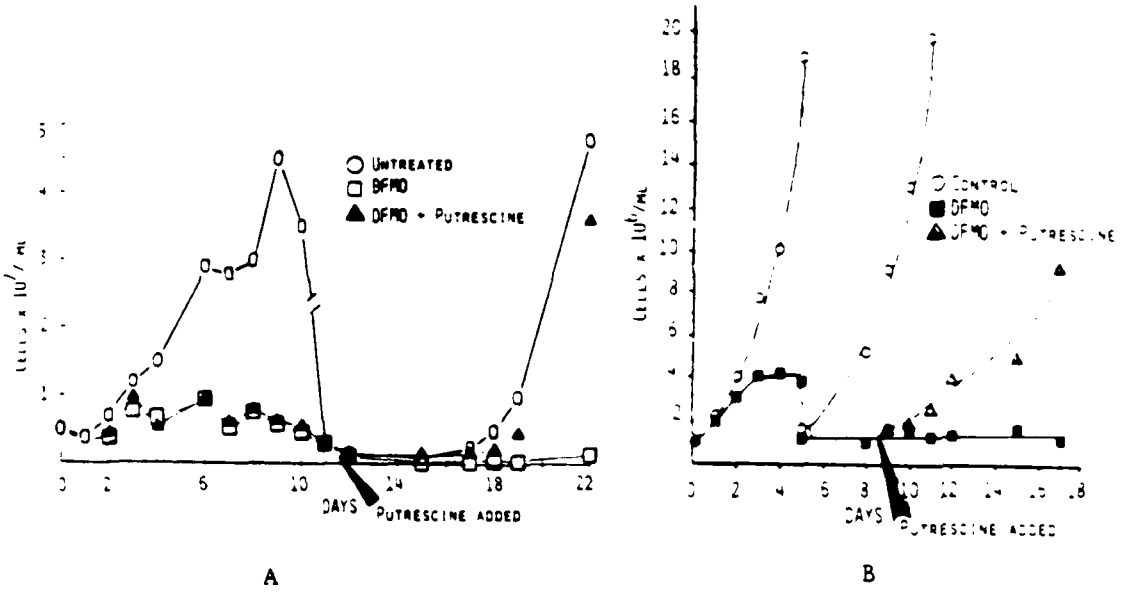

Fig. 2 Inhibition of growth by DFMO and its reversal by putrescine. (A) L. donovani, (B) L. braziliensis guyanensis

Table 8. Polyamine Levels* in species of Leishmania.

Organisms	Putrescine	Spermidine	Spermine
L. braziliensis guyanensis	7.5	11.2	3.2
L. mexicana amazonensis	8.5	18.5	3.0
L. major	14.0	15.5	3.0

*nmol/10^8 cells

These DFMO-induced changes in metabolite levels are similar to those reported for Trypanosoma b. brucei[4]. The effect of DFMO alone would appear to be cytostatic rather than cytocidal because non-dividing promastigotes can survive in culture for at least 11 days (Fig. 1 A-B). Although the precise mechanism by which DFMO induces cytostasis is unknown, it must be due to a depletion of polyamines and $T(SH)_2$, because its effect can be completely reversed by addition of putrescine (Fig. 2 A-B). These data suggest that it may be necessary to combine DFMO with a cytocidal drug like Bleomycin (Table 1) to effect a complete cure.

CONCLUSIONS

DFMO inhibits in vitro growth and in vivo replication of L. donovani and L. b. guyanensis, but not L. major or L. mexicana. The effect of DFMO alone upon L. braziliensis guyanensis infections is striking. This is the first time an oral, non-toxic, specific alternative to antimonials or Amphotericin B has been identified which is active against experimental mucocutaneous leishmaniasis.

Depending upon the species, DFMO can be synergistic, additive, or have no effect when combined with other drugs. DFMO and the antitumor antibiotic Bleomycin synergistically suppress experimental infections of L. donovani. When used in combination with Suramin, allopurinol riboside, Berenil, or Pentostam, DFMO has an additive effect against experimental visceral leishmaniasis.

Our in vitro data confirm these in vivo effects. DFMO significantly reduces putrescine and trypanothione levels in leishmania. Since leishmania and other trypanosomatids depend upon polyamine and trypanothione metabolism for survival (see Fairlamb, A.H., this volume), we think these should be actively pursued as potential targets for chemotherapy.

ACKOWLEDGMENTS

The authors thank Mr. Simon Paul and Mark Warshofsky for excellent technical assistance, Dr. Peter McCann (Merrell-Dow Research Institute) for supplying DFMO, Dr. Randolph Berens (University of Colorado Medical School) for supplying allopurinol riboside, Dr. Cyrus J. Bacchi (Pace University) for his helpful discussions, and Dr. Miklós Müller (Rockefeller University) for critical reading of the manuscript. This work was supported in part by grants from the Walter Reed Army Institute of Research, Merrell-Dow Institute of Research, the National Institutes of Health (JSK), and UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (AHF).

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ADDENDUM 2

IN: Leishmaniasis: The First Centenary (1885-1985). New Strategies for Control. D.T. Hart, editor. Plenum Publishing, New York City (in press). A NATO ASI Series Volume.

THE STEROLS OF LEISHMANIA PROMASTIGOTES AND AMASTIGOTES.

POSSIBLE IMPLICATIONS FOR CHEMOTHERAPY.

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INTRODUCTION

The major sterol identified [1] in the promastigotes of several Leishmania species is ergosta-5,7,24(28)-trien-3β-ol (11, Fig 1) and this is often accompanied by variable amounts of ergosta-5,7,22E-trien-3β-ol (ergosterol, 12). The presence of lanosterol (1) and a range of other trace sterols (2-10) in Leishmania [1,2], together with the results of labelling studies with [2-^{14}C]mevalonic acid [2-4], indicate that the major promastigote C_{28}-sterols are produced by the routes shown in Fig. 1. Similar metabolic routes are operative in many fungi which elaborate ergosterol (12) as the major sterol. When Leishmania promastigotes are cultured on a medium containing serum the cells contain considerable amounts of cholest-5-en-3β-ol (cholesterol) [1,2]. This cholesterol is derived from the serum lipoproteins and it does not appear to be metabolised by the promastigotes to C_{28} -sterols such as 11 or 12 [2].

A range of imidazole and triazole antifungal drugs have been designed which block the cytochrome P-450 dependent C-14 demethylation step in fungal ergosterol synthesis [5-7]. This causes the accummulation of 14α-methyl compounds such as lanosterol (1) and obtusifiol (3) with a concomittant decline in ergosterol (12). It is believed that this change in sterol composition results in a disruption of membrane function with consequential effects upon membrane enzymes and that this is the basis, at least in part, of the fungistatic and fungitoxic effects of these antifungal compounds [7].

The imidazole, ketoconazole, has been tested for activity against Leishmania species and shown to inhibit growth of L. mexicana mexicana promastigotes [4] and the propogation of amastigotes of three species of Leishmania in human and mouse macrophages [8-10]. Ketoconazole has also proved effective against visceral leishmaniasis in the golden hampster [11] and cutaneous and mucocutaneous lesions in man [12-16].

The similarity of sterol patterns between Leishmania and fungi prompted us to test the effect of the antifungal drug ketoconazole against L. mexicana

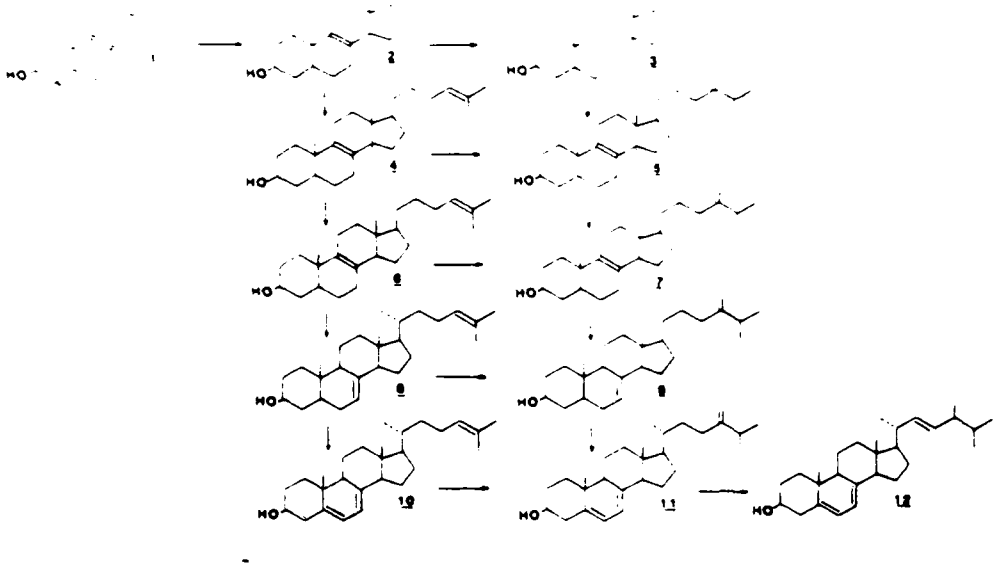


Fig. 1 Sterols of Leishmania species.

mexicana. Growth of both the promastigotes [2, 4] and amastigotes [3] was inhibited and 14α-methylsterols, particularly 4α,14α-dimethylcholesta-8,24-dien-3β-ol (2), accumulated and there was a decrease in the amount of C_{28}-sterols (9 and 11). It thus appears that the inhibitory effects of ketoconazole on Leishmania are probably the result of impaired sterol synthesis as in the case of fungi.

In the host animal, the Leishmania amastigotes reside in macrophage cells which have the capacity to synthesise cholesterol. Therefore we considered it important to determine the sterol composition of the amastigotes to ascertain if any sterols are present that possibly could be derived from the macrophages (or plasma lipoproteins). The uptake and utilisation of host sterols by the amastigote could have important implications for the successful use of imidazole drugs, or other sterol synthesis inhibiting drugs, against leishmaniasis.

MATERIALS AND METHODS

Amastigotes of L. braziliensis guyanensis (wild type, clones 1-A, 7-D, 8-2 and 8-3) and L. mexicana mexicana (WR227) were propagated in murine macrophage cells (J774.1) for 3-4 days [3]. The macrophages were lysed by two passages through a 30-gauge needle and the freed amastigotes were purified by differential centrifugation employing a Percoll gradient by the methods described previously [3].

Promastigotes of L. braziliensis guyanensis and L. mexicana mexicana were cultured as described elsewhere in Schneider's Drosphila medium or the RE III medium of Steiger and Steiger, respectively [2].

Samples of lyophilised cells were extracted twice with chloroform-methanol (2:1) and the volume of solvent reduced. The extract was then partitioned with petroleum ether to provide the sterol containing fraction [2, 7].

The sterols were analysed by GC-MS on a VG 70-70H mass spectrometer (source 220°C, 70ev, 4kV, 200μA) with a Finnigan Incos 2300 Data System and employing a fused silica bonded phase capillary column (BP-1, 25m x 0.25mm, on column injection at 50°C, then rapid heating to 250°C, then 4°C/min to 280°C). A few samples were analysed on a Hewlett-Packard 59085B GC-MS system fitted with a DB-1N (15m x 0.25mm) capillary column, initial temperature 70°C, then raised to 250°C at 20°C/min, then to 260°C at 4°C/min. Sterol identifications were based upon comparisons of mass spectra and GC retention times with those of authentic compounds.

RESULTS AND DISCUSSION

(a) Promastigotes

The promastigote forms of both L. braziliensis guyanensis and L. mexicana mexicana contained appreciable amounts of cholesterol (50-60% of total sterol). This will have been obtained from the serum in the growth media. The major C_{28}-sterol in L. mexicana mexicana was ergosta-5,7,24(28)-trien-3β-ol (11, about 30% of total sterol) and this was accompanied by ergosta-7,24(28)-dien-3β-ol (9, 7%). Small amounts of lanosterol (1, 4%) and 4α,14α-dimethylcholesta-8,24-dien-3β-ol (2, 2%) were also detected.

L. braziliensis guyanensis (clone 1-A) by contrast contained ergosta-5,7,22-trien-3β-ol (12, ergosterol, 30%) as the principal C_{28}-sterol. Also present was ergosta-7,24(28)-dien-3β-ol (9, 10%). The wild type and other clones had similar compositions, some with traces of 1 and 2, but in no case was more than a minor amount of ergosta-5,7,24(28)-trien-3β-ol (11) observed. L. braziliensis guyanensis differs from other species of Leishmania so far analysed [1] which although in several cases produce ergosterol (12) still have the 24-methylene compound 11 as the major of these two components. Sterol 11 is a probable precursor to ergosterol (12) (see Fig 1) and it thus appears that L. braziliensis guyanensis has a more evolved biosynthetic pathway leading to ergosterol as the predominant sterol. By contrast the L. mexicana mexicana promastigotes display little or no ability to introduce the ^{22}-bond required to produced ergosterol (12).

(b) Macrophage cells

As a prelude to determining the sterol composition of amastigotes it was first considered desirable to identify the sterols of the host macrophage cells. The two major components were readily identified from their characteristic GC retention times and mass spectra as cholesterol 90%, m/z 386, 321, 368, 313, 301, 275, 255, 213) and cholesta-5,24-dien-3β-ol (13, desmosterol, 10%, m/z 384, 369, 366, 351, 300, 271, 253, 213, 69). Cholesterol is the predominant sterol of mammalian tissues while demosterol (13) is an immediate precursor of cholesterol.

(c) Amastigotes

The recovered amastigotes of L. braziliensis guyanensis (Clone 1-A) gave a GC profile (Fig. 2) revealing a large amount of cholesterol (peak 251) and desmosterol (peak 268) but in addition other peaks (288 and 311) had the retention times of C_{28}-sterols. The identities of the cholesterol and desmosterol were confirmed by their mass spectra. However, the mass spectrum of peak 268 revealed a second co-chromatographing minor component with a molecular ion at m/z 398 and this, coupled with the retention time, allowed the probable identification of the compound as ergosta-5,22-dien-3β-ol (14).

The GC peak 288 had a mass spectrum displaying three molecular ions at m/z 400, 398 and 396 and a range of diagnostically valuable fragmentation ions. The library search by the data system provided an identification of the m/z 400 component as ergost-5-en-3β-ol (15, m/z 400, 382, 367, 315, 213) which was in accord with its retention time when compared to that of an authentic sample.

A second data system library search was then performed for the difference spectrum obtained by subtraction of the mass spectrum of 15 from the original mixture mass spectrum. The m/z 398 compound was easily identified as ergosta-5,24(28)-dien-3β-ol (16) because of the large fragment ion at m/z 314, This ion arises by a characteristic loss of part of the side chain (84 a.m.u.) due to the McLafferty rearrangement which occurs in sterols with a 24-methylene group in the side chain [17]. Also a large ion at m/z 271 was observed in the mass spectrum which is consistent with the presence of sterol 16.

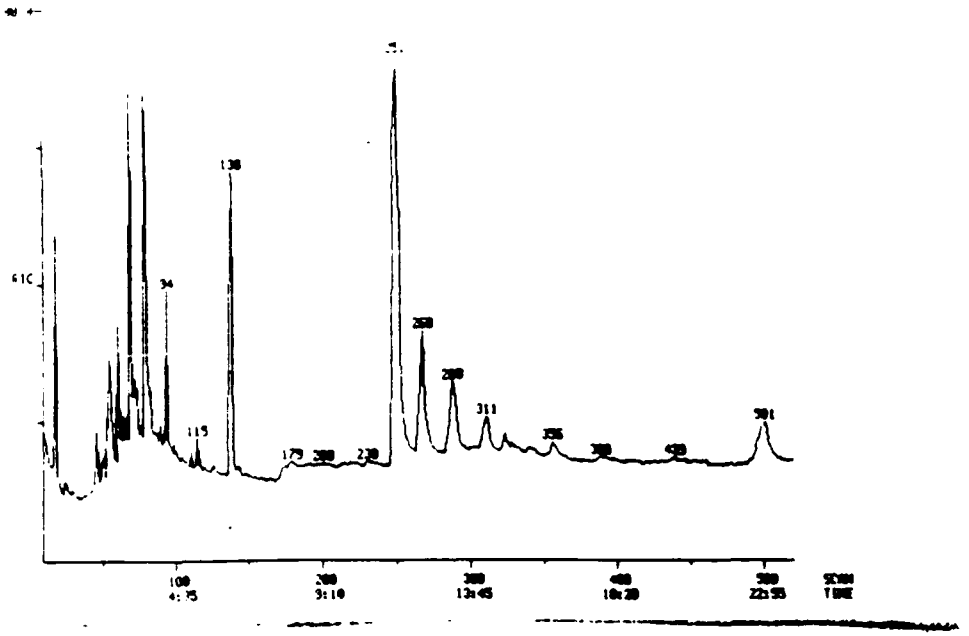
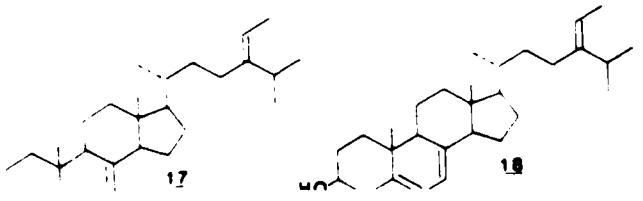


Fig. 2. GC-MS analysis of the sterols from amastigotes of L. braziliensis guyanensis.

A third library search was finally made for the difference spectrum remaining after subtraction of the spectra of both 15 and 16 from the original mixture mass spectrum. A remarkably good fit was obtained for the identification of the m/z 396 sterol as ergosterol (12, m/z 396, 363, 253, 211, 159, 147, 145) which was again consistent with its GC retention time.

Peak 311 in the GC analysis (Fig. 2) gave a mass spectrum revealing two compounds which were readily identified as ergost-7-en-3β-ol (m/z 400, 385, 273, 255, 229, 213) and ergosta-7,24(28)-dien-3β-ol (9, m/z 398, 383, 365, 314, 271, 255, 229, 227, 213). GC peak number 356, although a minor component, gave a good mass spectrum revealing it to be stigmasta-7,24(28)-dien-3β-ol (17) m/z 396, 314, 271, 253, 213) which was identified in larger amounts in the amastigotes of L. mexicana mexicana (see below). Amastigotes of the other clones of L. braziliensis guyanensis gave similar sterol profiles.

GC-MS analysis of the sterols obtained from L. mexicana mexicana amastigotes showed again cholesterol (40% of total sterol) but rather less desmosterol (1%) while ergosta-7,24(28)-dien-3β-ol (9) was the main C_{28}-sterol A most significant observation was that ergosta-5,24(28)-dien-3β-ol (16) comprised 7% of the mixture. The location of the ring system double bond at the C-5/C-6 position in 16 was confirmed by GC-MS analysis of the trimethylsilyl ether derivatives of the sterol mixture. The TMS-derivative of 16 gave a base peak at m/z 129 and an ion (rel. int. 14%) at m/z 341 $[M-129]^+$ which are typical fragment ions of a ^{5}-sterol TMS-ether [18]. In contrast to the sterols of L. braziliensis guyanensis there was no evidence for the presence of ergosta-5,22-dien-3β-ol (14) or ergost-5-en-3β-ol (15) in the amastigotes of L. mexicana mexicana. However, appreciable amounts of two C_{29}-sterols were found and identified as stigmasta-7,24(28)-dien-3β-ol (18, 9% of total sterols, m/z 412, 392, 314, 299, 271, 255, 213) and stigmasta-5,7,24(28)-trien-3β-ol (19, 10%, m/z 410, 395, 377, 351, 211, 159, 157, 145, 143). The mass spectra of the trimethylsilyl ether derivatives of the two C_{29}-sterols were consistent with these identifications.



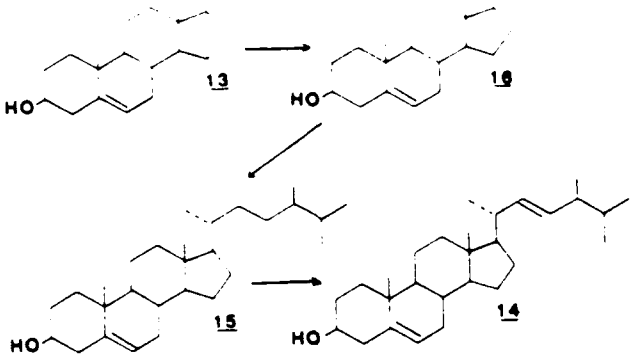


Fig 3. Suggested route for production of Δ^5 24-methylsterols from desmosterol (13) in Leishmania amastigotes.

The C_{29}-sterols have previously been observed in only small amounts in the sterol mixtures obtained from the promastigotes of most Leishmania species [1] although promastigotes of L. major contain an appreciable (5-8%) amount (unpublished observation). Sterols with the stigmastane skeleton are typically found in algae and higher plants and evidence is now emerging that 24-ethylsterols may play some essential role in plant cell growth [20]. The appearance of significant amounts of sterols 18 and 19 in the amastigote form of Leishmania thus presents an intriguing problem regarding their function. There must also be some signal which triggers increased C_{29}-sterol formation when the promastigote form develops into the amastigote in the host macrophage cell.

Equally significant is the presence of Δ^5-sterols alkylated at C-24 (14, 15, 16) in the amastigotes. This type of Δ^5-sterol has not been reported in the promastigotes which produce Δ^7- and $\Delta^{5,7}$-sterols, and like most fungi, Leishmania species may lack the ability to reduce the Δ^7-bond of $\Delta^{5,7}$-sterols to yield Δ^5-sterols [21]. We postulate that the Δ^5-sterols (14, 15, 16) are produced (Fig. 3) in the amastigote from desmosterol (14) that is a product of the host macrophage cell and which has been absorbed, together with cholesterol, by the amastigote. It is noteable that L. braziliensis guyanensis promastigotes produce ergosterol (12) with the 24-methyl Δ^{22}-side chain and the amastigotes can also apparently convert desmosterol to ergosta-5,22-dien-3β-ol (14) requiring the same side chain modifications. By contrast L. mexicana mexicana, which produces predominantly the 24-methylene sterol 11 in the promastigotes, must have little or no $\Delta^{24(28)}$-reductase or Δ^{22}-desaturase activities. Moreover, the amastigotes of this species can only apparently produce the 24-methylene derivative 16 from desmosterol and not the 24-methyl or Δ^{22}-compounds 14 or 15, respectively.

It has recently been shown that trace amounts of 24-methylsterols are essential for growth of yeast cells [22-24] and other fungi [25] while C_{27}-sterols (cholesterol, 5α-cholestanol) can satisfy a bulk requirement for membrane structures [26]. It is possible that C_{28}-sterols may play a similar essential role for cell division and growth in Leishmania amastigotes and this can be fulfilled by sterols 14-16 produced from host desmosterol. If this situation indeed pertains it has significant implications for the chemotherapy of leishmaniasis when employing drugs such as imidazole or allylamine antifungals [27] which block de novo sterol synthesis at an early stage in the sequence. The amastigotes may be able to survive the inhibition of endogenous de novo sterol synthesis by utilising absorbed host cholesterol for membrane functions and 24-alkyl sterols synthesised from absorbed desmosterol, for some other vital function where the C-24 substituted side chain structure is a critical requirement. Perhaps significantly, there have been a few reports of the ineffective use of imidazole drugs against cutaneous leishmaniasis in mice and man [28-30]. These possibilities are now being actively explored in our laboratories.

CONCLUSIONS

The promastigotes of L. braziliensis guyanensis and L. mexicana mexicana contain cholesterol, derived from the medium, and synthesise $^{5,7}C_{28}$-sterols. Ergosta-5.7,22-trien-3β-ol is the major product of L. braziliensis guyanensis but in L. mexicana mexicana ergosta-5,7,24(28)-trien-3β-ol predominates. There is no evidence of $^{5}C_{28}$-sterols in promastigotes.

The amastigote forms of these Leishmania species contain cholesterol and desmosterol which are products of the host macrophage cell. The amounts of $^{5,7}C_{28}$-sterols are diminished but the C_{29}-sterols stigmasta-7,24(28)-dien-3β-ol and stigmasta-5,7,24(28)-trien-3β-ol appear and account for about 20% of the sterol in the case of the L. mexicana mexicana amastigotes. $^{5}C_{28}$ sterols are also found in the amastigotes. L. mexicana mexicana contains ergosta-5.24(28)-dien-3β-ol but L. braziliensis guyanensis contain in addition ergost-5-en-3β-ol and ergosta-5,22-dien-3β-ol. We suggest that these sterols are synthesised in the amastigote by transmethylation of desmosterol which has been taken up from the host macrophage cell.

The possibilty that the C_{28}-sterols are essential for growth and cell division is discussed. The implications of utilisation of host sterol for membranes and for $^{5}C_{28}$ sterol production are considered in relation to the use of chemotherapeutic drugs which are effective through inhibition of de novo sterol synthesis.

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Acknowledgments: We thank Mark Prescott, Department of Biochemistry, Liverpool University, for the excellent GC-MS analyses.

Proceedings of the National Acad. Sci. March, 1988 (in press)

ADDENDUM 3

LR1: AN RNA VIRUS OF *LEISHMANIA*

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ABSTRACT

We report here the discovery of the first RNA virus in a kinetoplastid parasite. This virus, which we term LR1, is present in the promastigote form of the human pathogen *Leishmania braziliensis guyanensis* CUMC1-1A. LR1 RNA is about 6000 nucleotides, single stranded, and restricted to the cytoplasm. No homologous LR1 sequences are detected in genomic DNA. The viral RNA is associated with a 32nm diameter spherical particle that has a sedimentation coefficient of about 130S.

The presence of viruses in parasitic protozoa may be relevant to the resulting diseases and useful for molecular biological studies. DNA viruses have been found in *Amoeba* (1), double stranded RNA viruses have been found in *Giardia* (2) and *Trichomonas* (3) and circular DNAs have been detected in *Leishmania* (4). In addition, uncharacterized virus-like particles have been observed in Kinetoplastida (5,6). We report here the discovery and initial characterization of an RNA virus in *Leishmania braziliensis guyanensis*.

We examined 12 stocks of new world and one stock of old world *Leishmania* and found two that contain an abundant 6000 nucleotide nucleic acid (Fig. 1 and Table 1). This nucleic acid was absent from *Trypanosoma brucei* and *Trypanosoma cruzi* (data not shown). The high fluorescent intensity for the size of the nucleic acid from *L. braziliensis guyanensis* CUMC1-1A in ethidium bromide stained gels indicates that it has a high copy number. It is degraded by RNase and alkali but not by DNase (Fig. 2A) showing it is RNA. It was designated LR1 and examined in greater detail. The sensitivity to RNase was retained throughout salt concentrations up to 200 mM (Fig. 2B) indicating that the RNA is single stranded. RNA preparations reveal it has a density greater than 1.7 g/cm^3 indicating that the RNA is single rather than double stranded (7). A similar sized nucleic acid, designated LR2, was found in *L. braziliensis braziliensis* CUMC3 but its relationship to LR1 is unknown. LR1 is retained following passage of *L.b. guyanensis* CUMC1-1A through the sandfly (data not shown). A segment of LR1 RNA was cloned as a cDNA from gel purified 6000 nucleotide RNA (see note 12) and thus is probably from the viral genomic RNA rather than a transcript. The 5-30 cDNA clone hybridized to RNA from *L.b.*

guyanensis CUMC1-1A but not to RNA from *L.b. braziliensis* M2903 or *L. major* Jericho II (Fig. 1B), nor to *T. brucei* RNA (data not shown). Thus, the 5-30 cDNA originates from LR1 and has a restricted distribution among species and subspecies of kinetoplastids.

LR1 RNA is largely, if not exclusively, located in the cytoplasm. The 5-30 cDNA hybridizes strongly in northern blots to 6000 nucleotide RNA from the cytoplasmic fraction (Fig. 3, lane 2). A smear of hybridization corresponding to 2000 and 2300 nucleotide RNAs is also seen. There is no hybridization to RNA from the nuclear fraction in the experiment shown (Fig. 3, lane 1), however, sometimes slight hybridization to nuclear RNA was detected, probably due to cytoplasmic contamination since the nuclear fractions were not washed. Identical results are obtained whether the cells are ruptured using Triton-X100 or silica carbide grinding (8). Since the nuclear fraction also contains membrane components, these experiments indicate that little, if any, LR1 is associated with the cell membrane. The 5-30 cDNA did not hybridize to Southern blots of genomic DNA from *L.b. guyanensis* CUMC1-1A (data not shown). These experiments incorporated sensitivity controls detecting less than one copy per genome equivalent of the cDNA sequence. Thus, there is no genomic DNA copy of LR1 indicating that it is not a retrovirus.

When the SDS/proteinase K treatment of the cytoplasmic fraction was omitted, a smear of hybridization extending from the well to about the 9000 nucleotide region of the gel was observed (Fig. 3, lane 3). This suggests that LR1 occurs in a complexed form, perhaps as a particle. This possibility

was further examined using sucrose density gradients. Most 6000 nucleotide LR1 RNA is found in the fraction corresponding to a sedimentation coefficient of 130S (Fig. 4). There is also 6000 nucleotide LR1 RNA in the fraction corresponding to 40S, which may be free RNA. Electron microscopic analysis of the 130S fraction revealed spherical particles with a diameter of about 32 nm (Fig. 5). These particles were not present in comparable sucrose density gradient fractions from *L.b. guyanensis* M4147 which lacks the LR1 RNA. The coincidence of the LR1 RNA and the 32 nm particles in the same fraction and their apparent absence in cells lacking LR1 RNA suggests that the particles contain the LR1 RNA.

We conclude that LR1 is an RNA virus. The size and morphology of the particle, the size of the RNA, its maintenance during fly transmission, and its cytoplasmic location are consistent with the properties of other RNA viruses. Interestingly, the virus-like particles seen in *L. hertigi* (5) are also restricted to the cytoplasm. Infectivity experiments with LR1 are in progress. Nucleotide sequence analyses, to be presented elsewhere, reveal that the 5-30 cDNA clone from LR1 encodes a protein that is homologous to the L protein of vesicular stomatitis virus (VSV). The L protein is a subunit of the RNA-dependent RNA polymerase of VSV and is conserved among RNA viruses (9).

The effects of LR1 on the parasite are not obvious. There is as yet no correlation between the presence of LR1 and any disease characteristic, host range, or growth characteristic of *L.b. guyanensis*. The origin of the virus is also unknown, although it is intriguing that the sandfly, the vector for *Leishmania*, is also a known vector of RNA viruses. The initial

characterization presented here is insufficient to determine whether LR1 belongs to an existing class or constitutes a novel class of RNA virus. More importantly, LR1 is a potential transformation vector for *Leishmania* and possibly other kinetoplastids.

Fig. 1. LR1 viral RNA in *Leishmania*. Panel A. Ethidium bromide stained pulse field gel electropherogram prepared as described (10) showing the multicopy LR1. M, ligated λ DNA markers; lane 1, *L.b. guyanensis* M4147; lane 2, *L.b. guyanensis* CUMC1-1A; lane 3, *L.b. braziliensis* M2903. The LR1 band is indicated by the arrow. *Leishmania* promastigotes were grown as described (10). Panel B. Autoradiogram of a Northern blot showing hybridization of nick translated LR1 cDNA clone (prepared as described in 11) with total cellular RNA (as described in 12) from *L.b. guyanensis* CUMC1-1A but not *L.b. braziliensis* M2903 or *L. major*.

Fig. 2. LR1 contains single stranded RNA. A. LR1 is degraded by RNase A and NaOH but not DNase. LR1 RNA isolated by preparative gel electrophoresis was treated for 10 min at 37° with buffer alone, 10 μg/ml RNAse A in low salt buffer (20mM tris pH 7.5, 3mM $MgCl_2$), 47 U/ml RNase-free DNAse I (Pharmacia), a combination of both, or with 100mM NaOH. All samples were adjusted to the same salt concentration and electrophoresed in a 0.7% agarose gel, transferred to a Nytran membrane and hybridized to the 5-30 cDNA as described in Fig. 1. B. RNase sensitivity is unaffected by salt concentration. Gel purified LR1 RNA was treated with RNase A as described for panel A except that the NaCl concentration was varied as indicated above each lane. (+) RNase present; (-) RNase omitted.

Fig. 3. Subcellular localization of LR1. *L.b.guyanensis* CUMC1-1A cells were lysed with Triton X-100 as described (13) with some modifications. Cells (2 x 10^9/ml) were lysed in buffer containing 0.25M sucrose and 0.25% (v/v) Triton X-100 without heparin or cycloheximide. Nuclei were separated from cytoplasm by centrifugation and resuspended in storage buffer (50% glycerol, 20mM tris-HCl pH8, 75mM NaCl, 0.85mM DTT, 1mM EDTA) at 2 x 10^6 nuclei/μl. Nuclei, cytoplasm or intact cells were treated in some cases with 1% SDS and 20μg/ml proteinase K for 1 hr. at 65° to remove protein. A. Non-denaturing gel. Nuclei (4 x 10^7) (lane 1), cytoplasm (20 μl) (lane 2), untreated cytoplasm (20 μl, no SDS/proteinase K) (lane 3), and intact *L.b. guyanensis* CUMC1-1A cells (1 x 10^8) (lane 4) were electrophoresed in a 0.6% agarose gel. RNA was transferred to a Nytran membrane (12) after treatment with 50mM NaOH for 30min and hybridized to an LR1 cDNA riboprobe (14) in 50% formamide, 5xSSPE, 1x Denhardt's solution, 100μg/ml denatured salmon sperm DNA, 1% (w/v) Sarcosyl for 20 hrs at 65°. B. Denaturing gel. All fractions (lanes as in panel A) were electrophoresed in a formaldehyde containing 1.2% agarose gel (12). RNA was transferred and hybridized to the LR1 cDNA riboprobe as described in panel A.

Fig. 4. Sucrose gradient analysis of LR1 particle. One half ml cytoplasm was centrifuged through a 14 ml 15-30% linear sucrose gradient for 2.5 hr in a Beckman SW 40 rotor at 40,000 rpm. One ml fractions were collected and 20 μl aliquots treated with 1% SDS and 20μg/ml proteinase K for 1 hr at 65°, electrophoresed on a non-denaturing 0.6% agarose gel, transferred to a Nytran membrane and hybridized to the LR1 cDNA riboprobe as described in Fig. 3A.

Fig. 5. Electron micrograph of particles from the 130S sucrose gradient fraction. Fraction 3 (figure 4) was concentrated 5-fold by dialysis against dry Sephadex G200 (Pharmacia), deposited on parlodion coated grids, negatively stained with 0.3% uranyl acetate and 0.5 μl 0.015% octadecanol in hexanes (15), and visualized with a Philips EM-300. The scale bar is 100 nm.

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11. LR1 RNA was prepared from 1.35 x 10^{10} cells by lysis in 5ml of 1%(w/v) SDS, 1mg/ml proteinase K, 25mM EDTA for 1 hr at 50°. Chromosomal DNA was removed by potassium acetate precipitation and the supernatant isopropanol precipitated, resuspended and electrophoresed in a 0.7% agarose gel. The 6000 nucleotide band was excised from the gel, re-electrophoresed, electroeluted, and purified using glass milk (Bio 101, Inc.). Double stranded cDNA was prepared by the RNase H method [U. Gubler and B.J. Hoffman, *Gene* 25,263 (1983)] as modified below. LR1 RNA (25μl) was mixed and boiled with 10μl of random hexamers (Pharmacia, 0.3 $A_{260}/\mu l$) in the absence of Mg salts and incubated with 20U/ml MMLV reverse transcriptase (BRL) [G.F. Gerard, *Focus* 7,1 (1985)] in 80μl for 1 hr at 37°. Second strand synthesis was performed for 1 hr at 14° followed by 1 hr at room temperature. Ends were filled with the Klenow fragment of DNA polymerase I (BRL) and BamHI linkers added. The cDNA was ligated into Bluescribe vector, transformed into DH5α cells, and LR1 cDNAs determined by colony hybridizations [(M. Grunstein and D.S.

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Hogness, *Proc. Natl. Acad. Sci. USA* 72,3961 (1975)] to radiolabelled first strand synthesis product.

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14. LR1 5-30 cDNA riboprobe was prepared using T7 RNA polymerase. One μg of cDNA plasmid (linearized with SalI) was transcribed under conditions recommended by Stratogene for high specific activity transcripts.

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16. We thank R.A. Sutherland for technical assistance, B. Byers for use of the electron microscope, P.G. Lawyer for the transmission of *Leishmania* through sandflies, J. Feagin for *L. major* RNA, S. Reed for *Leishmania* stocks, S. Klebanoff for help and encouragement and V. Rothwell for critical review of the manuscript. This work was supported by NIH grants AI24771 (to K.S.) and AI16282 (to J.K.) and by the World Health Organization's Special Programme for Research and Training in Tropical Diseases. P.T. was supported by the Rockefeller Foundation and Training Grant T32 HD07233 and K.S. is the recipient of a Special Fellowship from the Burroughs Wellcome Fund.

Table 1. *Leishmania* isolates tested for LR1 RNA.

Species	WHO Designation	RNA
L. braziliensis guyanensis	MHOM/SR/81/CUMC1-1A	LR1
L. braziliensis guyanensis	MHOM/BR/75/M4147*	-
L. braziliensis braziliensis	MHOM/PE/83/CUMC3	LR2
L. braziliensis braziliensis	MHOM/BR/75/M2903	-
L. braziliensis braziliensis	MHOM/BR/00/LTB300*	-
L. braziliensis braziliensis	MHOM/BR/75/M2904	-
L. braziliensis panamensis	MHOM/PA/71/LS94*	-
L. braziliensis panamensis	MHOM/BZ/00/470	-
L. donovani chagasi	MHOM/BR/82/BA-3	-
L. mexicana amazonensis	MHOM/BR/76/Josepha	-
L. mexicana amazonensis	MHOM/BR/80/Maria	-
L. mexicana mexicana	MHOM/BZ/82/BEL21*	-
L. major	MHOM/IL/67/Jericho II	-

L. major was provided by Dr. Steven Reed, Seattle Biomedical Research Institute, Seattle, Wa., *L.b. braziliensis* M2904 was provided by Dr. Jan Keithly, Cornell University Medical College, N.Y. Other strains were described previously (10). The * denotes World Health Organization Reference Strains.

M 1 2 3 M

LR1

Fig 1

LBG
LBB
L MAJ

6KB–[redacted]

PROBE: 5–30

Fig. 2

Fig. 3

B)

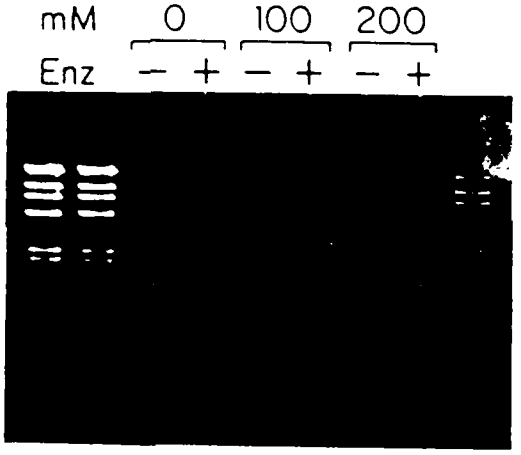

Fig. 3

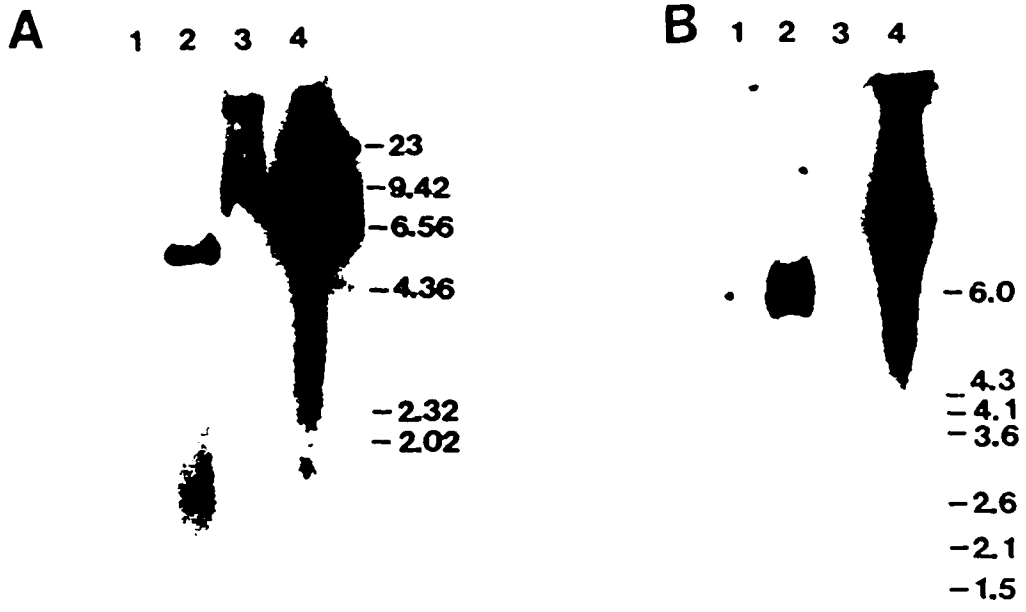

Fig. 4